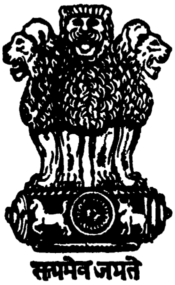

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I. STUDIES ON THE REVERSIBLE INACTIVATION OF PAPAIN AND CATHEPSIN.

By ARNULF PURR.

From the Cancer Research Laboratories, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.

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The natural activation of cathepsin and papain by sulphydryl compounds acquired increased physiological interest when it became known [Purr, 1933] that the combination, vitamin C-Fe″, can activate not only arginase but also cathepsin. Later, Maschmann and Helmert [1934] found a similar activation of papain by the vitamin C-Fe″ complex.

The activation of these two proteinases by such widely dissimilar types of compounds made it of interest to investigate further the mechanism of these activations. The experiments here reported show much promise, contributing not only to our knowledge of the nature of intracellular proteolysis, but also to our understanding of the part played by vitamin C in the regulation of cellular metabolic processes.

Since the initial activities of papain and cathepsin vary considerably for different preparations, it was necessary to devise methods for obtaining entirely inactive enzyme preparations. For comparative studies in enzyme activation, it was essential that these inactive preparations should retain the property of being completely reactivated upon addition of the proper activator.

Reversible inactivators of papain.

I. *Alloxan inactivation and reactivation.* Papain extract was prepared from a commercial Merck papain powder by 6 hours' extraction with 10 parts of water. After centrifuging, 4 ml. of the clear fluid were allowed to stand for 30 minutes at 30° and p_H 7 with 40 mg. of cysteine hydrochloride dissolved in 4 ml. of water. To the thus fully activated enzyme solution were then added 80 mg. of alloxan in 4 ml. of water, and the reaction mixture was incubated for 1 hour at 30° and p_H 7. The volume was brought to 20 ml., and the papain activity determined on 10 ml.; H_2S was bubbled through the remaining 10 ml. for 1 hour at p_H 7, and the degree of reactivation determined. (If cysteine be used for reactivation, it is necessary to use a large excess, 50 mg., because of the presence of excess alloxan.) The measurement of enzymic activity was carried out by adding 5 ml. of 8 % gelatin, 2 ml. of M acetate buffer of p_H 5 and sufficient water to bring the volume up to 25 ml. 10 ml. of this were analysed at once for free amino-groups, and a second 10 ml. after 1 hour's incubation at 30°. The increase of free amino-groups, determined by the Van Slyke method, constitutes a measure of the enzymic activity, the results being expressed in ml. of 0·05N KOH.

For the determination of the initial activity before inactivation, 2 ml. of the papain extract, prepared as described above, were mixed with 5 ml. of 8 % gelatin, 2 ml. of *M* acetate buffer of p_H 5 and sufficient water to bring the total volume up to 25 ml. Amino-group determinations were carried out immediately and after 1 hour's incubation at 30° on 10 ml. portions of the mixture, exactly as previously described. For the determination of full activity before inactivation, 2 ml. of the original papain extract were fully activated by passing a stream of H_2S for 1 hour at 30°. The rest of the treatment was identical with that just described.

II. *Methylglyoxal inactivation and reactivation.* The experimental details of this experiment were identical with those described for alloxan, except that for inactivation 48 mg. of methylglyoxal in 5 ml. of water were used in place of the alloxan. The methylglyoxal was prepared by distillation of dihydroxyacetone with dilute sulphuric acid according to the method of Neuberg *et al.* [1917]. Since the same papain extract was used, the initial and full activities before inactivation are the same as those in the case of alloxan.

Table I. *Reversible inactivation of papain by alloxan and methylglyoxal.*

Reversible inactivator	Papain activity before inactivation: Initial activity	Papain activity before inactivation: Full activity	Papain activity after inactivation	Reactivation: Cysteine	Reactivation: H_2S
	0·80	3·54	—	—	—
Alloxan	—	—	0·0	3·50	3·65
Methylglyoxal	—	—	0·0	—	3·60

The results given in Table I (as NH_2 increase in ml. 0·05*N* KOH) show not only that alloxan and methylglyoxal completely remove the slight initial activity present in the papain preparation, but also that the enzyme, after inactivation by these compounds, may be completely reactivated, showing that the enzyme itself has not been damaged. In separate experiments with the nitroprusside test for SH groups, it was found that the inactivation is connected with a disappearance of the SH reaction. This finding demonstrates the dependence of papain activity on the presence of SH groups.

The inactivation of papain by alloxan and methylglyoxal differs sharply from that brought about by iodoacetic acid. The inhibition produced by the latter, which is irreversible, will be discussed later in more detail.

III. *The system "aerobic dehydrogenase-peroxidase."* The application of this system to the reversible inactivation of papain is suggested by the illuminating work of Thurlow [1925], who found that the system aerobic dehydrogenase-peroxidase catalyses the formation of H_2O_2 from molecular oxygen. An aerobic dehydrogenase, such as xanthine oxidase, is able, in the presence of hypoxanthine, to make molecular oxygen directly available as a hydrogen acceptor, and, in the presence of excess oxygen, to produce H_2O_2. As Thurlow found, the quantity of H_2O_2 formed is very small at the beginning of the experiment, but as soon as the greater part of the hypoxanthine is converted into xanthine, the quantity increases remarkably. Bach and Chodat [1903] have defined as an oxygenase a system which is able to convert molecular oxygen directly into H_2O_2. Thurlow showed that the system xanthine dehydrogenase-hypoxanthine falls within the classification of oxygenases.

In contrast to this are the anaerobic dehydrogenases, such as lactic dehydrogenase, which do not possess the ability to react directly with molecular oxygen

Only the addition of a small quantity of methylene blue enables the system to react with molecular oxygen. The methylene blue acts as an intermediate carrier (hydrogen acceptor) between oxygen and the dehydrogenase substrate.

Peroxidase is an enzyme which activates the oxygen in the H_2O_2 molecule. This active condition of the oxygen enables it to transfer its oxidation potential very easily to other substances, which themselves are oxidisable.

If such a system (aerobic dehydrogenase + substrate + peroxidase) is added to a papain preparation, and care is taken that the oxygen of the air comes into intimate contact with the solution, a complete inactivation of the papain is obtained. This inactivation occurs only when all the components mentioned are present, the separate components being without effect on the enzymic activity.

The procedure for the inactivation was as follows: 30 mg. of milk xanthine oxidase (prepared by the method of Dixon and Thurlow [1924]), 1 mg. of hypoxanthine and 10 mg. of milk peroxidase [Elliott, 1932] were dissolved in 2 ml. of water and added to 2 ml. of the papain extract previously described. 1 ml. of $M/10$ phosphate buffer of p_H 7 was added. Air was then bubbled through the solution for 3 hours, after which 5 ml. of 8 % gelatin, 2 ml. of M acetate buffer of p_H 5 and sufficient water to bring the total volume up to 25 ml. were added. Amino-determinations were carried out immediately, and after 1 hour's incubation at 30°, on 10 ml. portions. The results are shown in Table II as NH_2 increase in ml. of $0 \cdot 05 N$ KOH. A similar inactivated reaction mixture was treated with H_2S for 1 hour at p_H 7 and the degree of reactivation obtained measured as previously described. The determination of initial and full activity of the papain before inactivation was carried out as described under Table I.

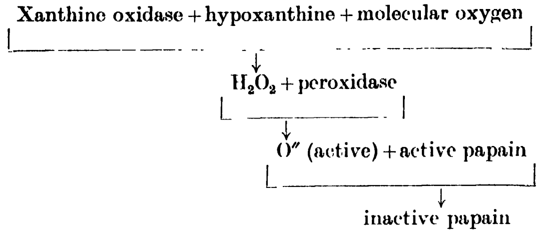
The following control experiments were carried out: (*a*) 2 ml. of papain extract were diluted with 2 ml. of water and 1 ml. of $M/10$ phosphate buffer of p_H 7; air was then bubbled through for 3 hours and the papain activity determined as described above; (*b*) same as (*a*) except that 1 mg. of hypoxanthine was added; (*c*) same as (*a*) except that 1 mg. of hypoxanthine and 30 mg. of milk xanthine oxidase were added; (*d*) same as (*a*) except that 30 mg. of milk xanthine oxidase and 10 mg. of milk peroxidase were added.

Table II. *Reversible inactivation of papain by respiration ferments.*

Reversible inactivator	Papain activity before inactivation: Initial activity	Papain activity before inactivation: Full activity	Papain activity after inactivation	Reactivation: Cysteine	Reactivation: H_2S
	0·90	3·50	—	—	—
D + Hy. + Per. + O_2	—	—	0·05	3·50	3·40
O_2 (3 hours)	—	—	0·90	—	—
Hy. + O_2	—	—	0·90	—	—
Hy. + D + O_2	—	—	0·85	—	—
D + Per. + O_2	—	—	0·90	—	—

D = aerobic dehydrogenase; Hy. = hypoxanthine; Per. = peroxidase.

As shown in Table II, the reversible inactivating effect on papain is obtained only by the simultaneous action of xanthine oxidase (aerobic dehydrogenase), hypoxanthine (substrate for the dehydrogenase), peroxidase and excess oxygen. From these findings, which for the first time demonstrate the dependence of intracellular proteolysis on the respiration ferments, it may be concluded that the following intermediary steps are responsible for the papain inactivation:


Xanthine oxidase + hypoxanthine + molecular oxygen
H_2O_2 + peroxidase
O″ (active) + active papain
inactive papain

IV. *Hydrogen peroxide.* Preliminary experiments on the reversible inactivation of papain with hydrogen peroxide gave satisfactory results. A method was therefore developed which permits the preparation of large quantities of inactive papain in a stable, pulverisable form.

To an aqueous solution of 5 g. of papain (Merck) in 100 ml. are added 3 ml. of 30 % H_2O_2, and the mixture is allowed to stand 1 hour at 30°. The enzyme is then precipitated by addition of 600 ml. of 95 % alcohol, centrifuged, and the residue washed twice with 95 % alcohol. After the final centrifuging, the residue is redissolved in 100 ml. of water (any insoluble matter is removed by centrifuging) and treated again with H_2O_2 and alcohol as described above. This procedure is repeated five times. The final precipitate is dried *in vacuo* over P_2O_5 and powdered.

For reactivation experiments, the inactive powder was extracted with 40 parts of water and centrifuged. 1 ml. of the liquid (comparable in activity with 2 ml. of crude papain extract before inactivation) was brought up to 5 ml. with water, mixed with 20 mg. of cysteine HCl in 2 ml. and allowed to activate for 30 minutes at p_H 7. The measurement of enzymic activity, as well as the determination of initial and full activity before inactivation, were carried out as previously described.

Table III. *Reversible inactivation of papain by hydrogen peroxide.*

Reversible inactivator	Papain activity before inactivation		Papain activity after inactivation	Reactivation	
	Initial activity	Full activity		Cysteine	H_2S
	0·80	3·30	—	—	—
H_2O_2	—	—	0·0	3·35	3·40

Table III shows that papain inactivated by hydrogen peroxide can be fully reactivated, just as is the case with the other inactivators described in this paper. The method described for the inactivation of papain by hydrogen peroxide possesses the advantage of being easily performed. The technique is therefore peculiarly adapted to the preparation of large amounts of papain, so that comparative activation studies on this enzyme are made much simpler.

Reversible inactivation of cathepsin.

I. *Alloxan.* Rat liver was used as the source of cathepsin. Liver from well-bled rats was finely ground in a micro-mincing machine (Leitz). The "brei" thus obtained was suspended in 10 parts of 90 % glycerol and used directly for the enzyme studies.

To obtain an estimate of the initial activity, 5 ml. of this suspension were treated with 5 ml. of 8 % gelatin (Kahlbaum), 1 ml. of N acetic acid and sufficient water to bring the volume up to 25 ml. (final $p_H = 4$). By means of the Van Slyke method, amino-nitrogen was estimated immediately, and again after 24 hours' incubation at 30°, on 10 ml. portions of the reaction mixture. As before, the increase in amino-nitrogen, expressed in ml. of $0{\cdot}05N$ KOH, indicates the cathepsin activity. A second portion of the glycerol suspension was fully activated by allowing 5 ml. to stand for 15 minutes at 30° and p_H 7 with 20 mg. of cysteine hydrochloride and 30 mg. of $FeSO_4, 7H_2O$ in 3 ml. of water. The determination of the full activity then followed exactly as described for the initial activity.

To demonstrate the inactivating effect of alloxan, 5 ml. of the glycerol suspension were fully activated by cysteine, then allowed to stand for 1 hour at 30° and p_H 7 with 30 mg. of alloxan, after which activity determinations were carried out as before. A second portion of the glycerol suspension was fully activated by cysteine, inactivated by alloxan and finally reactivated by passing H_2S for 1 hour at p_H 7. The results obtained are shown in Table IV.

II. *Methylglyoxal.* The experimental procedure was the same as that described for alloxan, except that 30 mg. of methylglyoxal were used in place of the alloxan. The results are shown in Table IV.

III. *Iodoacetic acid.* 5 ml. of glycerol suspension were fully activated by keeping with 26 mg. of cysteine hydrochloride in 2 ml. of water for 30 minutes at 30° and p_H 7. A second 5 ml. portion was first activated, then inactivated by allowing it to stand for 30 minutes with 15 mg. of neutralised iodoacetic acid. Attempts were made to reactivate similarly inactivated reaction mixtures by treatment with 3-fold quantities of cysteine hydrochloride (30 minutes at 30°) and with H_2S (1 hour). Activity determinations were made on all reaction mixtures by the usual procedure. The results are shown in Table IV.

Table IV. *Effect of alloxan, methylglyoxal and iodoacetic acid on cathepsin activity.*

Inactivator	Initial activity	Full activity	SH test* before inactivation	¼ hour inactivation		1 hour inactivation		Reactivation	
				Enzymic activity	SH test	Enzymic activity	SH test	H_2S	Cysteine
None	0·50	1·70	+ +	—	—	—	—	—	—
Alloxan	—	—	+ +	0·8	+	0·0	—	1·50	1·20
Methylglyoxal	—	—	—	—	—	0·0	—	1·60	—
Iodoacetic acid	—	—	+ +	0·0	+ +	0·0	+	0·0	0·0

* Nitroprusside colour reaction.

As shown in Table IV, alloxan and methylglyoxal differ from monoiodoacetic acid in their mode of action on cathepsin. Cathepsin inactivated by the latter cannot be reactivated by direct addition of cysteine or H_2S, in contrast to alloxan- or methylglyoxal-inactivated cathepsin. The same difference is shown by a study of the SH colour reaction (nitroprusside test). It was found that a parallelism exists between the disappearance of sulphydryl from the cathepsin (and also papain) complex and the reversible inactivation by alloxan, methylglyoxal, H_2O_2, or by the system aerobic dehydrogenase-substrate-peroxidase. In the case of iodoacetic acid inactivation, however, no relation exists between loss of catheptic activity and loss of SH. Even after the enzyme has been completely inactivated, a strong SH test is obtained, which, however, eventually disappears. This irreversible inactivation of papain and cathepsin by iodoacetic acid does not involve a destruction of the enzyme complex. If iodoacetic acid-inactivated cathepsin or papain is treated with 80 % alcohol, according to the procedure of Maschmann and Helmert [1933], the enzyme may be reactivated practically to its original activity with cysteine.

A series of experiments was carried out on the inactivation of cathepsin by iodoacetic acid, to determine the effect of varying the order of adding the various reagents. (*a*) 6 ml. of 1:10 glycerol extract of acetone-ether-dried pig liver were activated with 25 mg. of cysteine hydrochloride in 2 ml. of water by keeping for 30 minutes at 30° and p_H 7. The mixture was then treated with 25 mg. of iodoacetic acid in 2 ml. of water and again allowed to stand for 1 hour. (*b*) The same quantities of cysteine hydrochloride and iodoacetic acid were added simultaneously to 6 ml. of glycerol extract and kept for 1 hour at 30° and p_H 7. (*c*) The same quantities of cysteine hydrochloride and iodoacetic acid were mixed and kept for 1 hour at 30° and p_H 7. 6 ml. of fully activated glycerol extract were then added. (*d*) To 6 ml. of fully activated glycerol extract were added 5 ml. of 8 % gelatin and then 25 mg. of iodoacetic acid in 2 ml. of water; 1 ml. of *N* acetic acid and sufficient water were then added to bring the volume to 25 ml. (p_H 4). The mixture was incubated for 24 hours at 30°. The various reaction mixtures were then treated with H_2S or cysteine hydrochloride (6 × 26 mg.) for 1 hour and, after addition of the appropriate reagents, tested for possible enzyme reactivation.

It was found that the order of adding the various reagents is immaterial. In each case the enzyme was found to be completely inactivated, and no reactivation was obtained with either H_2S or cysteine. The same results were obtained with papain. It should be mentioned that the inhibition with iodoacetic acid appears not to be a time reaction, but is effective immediately, even when excess SH groups are present.

The comparison of the two inactivation effects, the one reversible (alloxan *etc.*) and the other irreversible (iodoacetic acid) gives rise to conjecture as to the active groupings in papain and cathepsin. The reversible inactivation seems to be connected with a destruction of the SH groups in the papain and cathepsin complexes, and is probably related to interference in the reaction of enzyme with activator. The irreversible inactivation, on the other hand, appears to be concerned not only with an action on the SH groups, but also on a second essential grouping, which seems to be even more important to the enzyme than the SH groups. According to Willstätter *et al.* [1922] an enzyme consists of a colloidal carrier and an active group. If this is correct, then it appears probable that the papain and cathepsin molecules are equipped with at least two active groups, one of which may safely be specified as the SH group.

IV. *Reversible inactivation of liver cathepsin by organic solvents.* The following method was adopted for the preparation of large quantities of inactive cathepsin, as required for comparative activation experiments on this enzyme. Fresh pig's liver was ground in a micro-mincer and suspended in 15 parts (by weight) of absolute acetone. After shaking continuously for 1 hour the suspension was filtered by suction. This procedure was repeated three times. The residue was then extracted three times with 15 parts of an acetone-ether mixture (1:1), each extraction requiring 1 hour. Finally the residue was extracted three times with 15 parts of ether. After drying, the residue was finely ground and passed through a sieve and then extracted for 12 hours with 10 parts by weight of 85 % glycerol. After filtration the clear liquid contained the inactive cathepsin.

If, as sometimes happens, the cathepsin is not yet entirely inactive, excess 95 % alcohol may be added to the glycerol extract until no further precipitation occurs. Ordinarily 10–15 volumes of alcohol are required. After 6 hours' standing, the precipitate is filtered or centrifuged off and dried with ether. A glycerol extract is then prepared from the dried powder as previously described. The enzyme is now completely inactive. Table V shows the initial and full activity of the enzyme at the various stages of the procedure just described.

Table V. *Reversible inactivation of cathepsin by organic solvents.*

		Activity before inactivation		Activity after inacti-vation	Reactivation	
Treatment	Organ	Initial	Full		H_2S	Cysteine
Glycerol suspension 1 : 10 (fresh liver)	Pig liver	1·0	2·40	—	—	—
Glycerol extract 1 : 10 from dried liver after treatment 7 × with acetone, 3 × acetone-ether (1 : 1) and 3 × ether	,,	—	—	0·6	1·60	1·90
Glycerol extract 1 : 10 after alcohol treatment of the above glycerol extract	,,	—	—	0·00	1·10	1·00
Glycerol suspension 1 : 10 from fresh liver	Rabbit liver	0·90	2·20	—	—	—
Glycerol extract 1 : 10 from dried liver after treatment 4 × with acetone, 3 × acetone-ether (1 : 1) and 3 × ether	,,	—	—	0·00	1·0	1·00

The final treatment with alcohol appears not to be necessary in the case of rabbit liver, where only a few minutes elapsed between the death of the animal and the first treatment of the liver mince with acetone.

CONCLUSIONS.

I. *Reversible inactivation of papain.*

1. A method is described for the reversible inactivation of papain by means of alloxan, methylglyoxal, hydrogen peroxide and with the system milk xanthine oxidase + hypoxanthine + milk peroxidase. From the observation that the reversible inactivation of papain proceeds parallel with the disappearance of the SH colour reaction with sodium nitroprusside it is concluded that the destruction of the SH groups in the papain complex (the same applies to the cathepsin complex) through the reagents mentioned is responsible for the reversible inactivation.
2. A relation between typical respiration ferments and intracellular proteolysis was shown by the reversible inactivation of papain by the system aerobic dehydrogenase + hypoxanthine + peroxidase.
3. A method is described for the preparation of large quantities of reversibly inactivated papain in stable form.

II. *Reversible inactivation of cathepsin.*

1. Cathepsin is also reversibly inactivated by alloxan or methylglyoxal. The loss of enzymic activity runs parallel with the loss of SH.

2. On the basis of the reversible and irreversible inactivations of papain and cathepsin the possibility is suggested that the two enzyme systems require at least two active groups for their activity, of which groups one can be said with certainty to be an SH group.

3. A method is described for the preparation of large quantities of inactive cathepsin.

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REFERENCES.

Bach and Chodat (1903). *Ber. deutsch. chem. Ges.* **36**, 606.
Dixon and Thurlow (1924). *Biochem. J.* **18**, 971.
Elliott (1932). *Biochem. J.* **26**, 10.
Maschmann and Helmert (1933). *Z. physiol. Chem.* **220**, 199.
—— —— (1934). *Z. physiol. Chem.* **223**, 127.
Neuberg, Färber, Levite and Schwenk (1917). *Biochem. Z.* **83**, 262.
Purr (1933). *Biochem. J.* **27**, 1703.
Thurlow (1925). *Biochem. J.* **19**, 175.
Willstätter, Graser and Kuhn (1922). *Z. physiol. Chem.* **123**, 45.

II. THE ACTIVATION PHENOMENA OF PAPAIN AND CATHEPSIN.

By ARNULF PURR.

From the Cancer Research Laboratory, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.

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THE discovery [Purr, 1933] of the activating effect of the vitamin C—Fe″ complex on arginase and cathepsin demonstrates that a product of plant cellular metabolism may, like sulphydryl compounds (*e.g.* glutathione), exercise a regulatory action on animal protein metabolism. Later investigations by Maschmann and Helmert [1934], as well as the present work, show that this activating effect of vitamin C—Fe″ is not limited to animal proteases, but extends also to papain. The latter is the protease of many plants and appears to be the complete counterpart of cathepsin, the protease of most animal organs and leucocytes. According to our present knowledge, a close relationship exists between the proteolytic enzyme systems of the plant and animal worlds.

The vitamin C—Fe″ complex is a physiological product which resembles HCN in its activating effect on papain and cathepsin. The discovery of this new activator complex has cast considerable doubt on the widely held idea that sulphydryl compounds function as specific activators of these proteinases. Purr and Weil [1934] have likewise found evidence that sulphydryl may not be a specific activator of arginase. The clarification of these questions is of considerable physiological significance because of the fact that the newly-found activator is a vitamin.

The present investigation deals with the activation phenomena of papain and cathepsin. It is a continuation of a series of studies which has as its goal the explanation of the relation between intracellular enzymes and vitamins. A further aim is to clarify our knowledge of the mechanism of enzyme activation, so that differences between normal and cancerous enzymic processes may more readily be detected. It appears quite probable that the proteinases of specific organs (*e.g.* liver) will differ in some essential points (perhaps in activation mechanism) from those of pathological neoplasms, which have been designated by Waldschmidt-Leitz *et al.* [1933] as unspecific organs with respect to their enzymic equipment.

Activation of papain.

For comparative activation studies, a papain preparation was used which had been reversibly inactivated with H_2O_2 according to a procedure described by Purr [1934]. The use of such an inactivated preparation makes the results obtained independent of the variations in initial activity which are characteristic of natural papain. 1 g. of inactive papain was extracted by standing overnight in the ice-box with 50 ml. of water. After filtration and addition of a drop of toluene the extract may be preserved for a long time in the ice-box.

The initial activity of this extract was determined as follows. 0·20 ml. of papain extract was treated with 20 mg. of $FeSO_4, 7H_2O$ in 1 ml. of H_2O, 5 ml.

of 8 % aqueous gelatin, 2 ml. of acetate buffer of p_H 5 and sufficient water to bring the total volume to 25 ml. 10 ml. were removed immediately and 10 ml. after an incubation period of 20 hours at 30° for analysis. The increase in free amino-groups was estimated by the Van Slyke method, the results being expressed in ml. of 0·05 N KOH.

By this procedure, the following activation experiments were carried out. (*a*) 0·20 ml. of papain extract was incubated for 15 minutes at p_H 7 with 20 mg. cysteine hydrochloride in 2 ml. of water and 20 mg. of $FeSO_4, 7H_2O$ in 1 ml. of water; (*b*) 0·20 ml. of papain extract was treated for 1 hour at 30° with 5 ml. of $M/5$ KCN at p_H 7; (*c*) 0·20 ml. of papain extract was incubated for 1 hour at 30° and p_H 7 with 7 mg. vitamin C in 2 ml. H_2O and 20 mg. $FeSO_4, 7H_2O$ in 1 ml. of H_2O; (*d*) same as (*c*) except that 20 mg. of pyruvic acid were substituted for the vitamin; (*e*) same as (*c*) except that 20 mg. of succinic acid were substituted for the vitamin.

The reaction mixtures were then carried through the same procedure as described for initial activity. The entire series was repeated (except for the HCN experiment) in the absence of iron salt. The results are shown in Table I.

Table I. *Reactivation of inactive papain.*

Reactivator	Activity (ml. 0·05 N KOH) Without Fe″	With Fe″	Time of incubation (hours)
None	0·00	0·00	20
Cysteine	4·85	5·00	20
HCN	3·10	—	20
Vitamin C	0·00	0·05	20
„	—	0·80	70
Pyruvic acid	0·00	0·05	20
Succinic acid	0·00	0·00	20

It is evident that neither vitamin C nor the vitamin C—Fe″ complex is able to reactivate the purified, reversibly inactivated papain used. A possible explanation for this finding, which is contrary to the results obtained by Maschmann and Helmert [1934], is suggested by the appreciable activation obtained on longer incubation (70 hours). It appeared likely that certain contaminating substances, which were removed by the purification and inactivation process, might be responsible for the variations in the results.

The experiment was therefore repeated using a 1:10 aqueous extract (6 hours' extraction) of commercial unpurified papain (Merck). The experimental details were similar to those described for Table I, except that 2 ml. of the papain extract were used. The results are shown in Table II.

Table II. *Activation of unpurified commercial papain.*

Activator	Activity in ml. 0·05 N KOH Without Fe″	With Fe″
None	0·70	0·71
Cysteine	5·10	5·20
HCN	5·65	—
Vitamin C	0·80	2·41
Pyruvic acid	2·10	2·20
Succinic acid	0·65	0·75

In agreement with Maschmann and Helmert's [1934] results, it was found that the vitamin C—Fe″ complex activates unpurified commercial papain. The pyruvic acid—Fe″ complex likewise produces a similar degree of activation. In the absence of iron salts, vitamin C produces no activation, while under the same circumstances pyruvic acid produces nearly as high an activation as with iron.

The failure of the vitamin C—Fe″, and the pyruvic acid—Fe″ complexes to activate purified inactive papain led to the conclusion that these combinations are able to activate papain only indirectly. Observations made during a search for a possible contaminating substance, which might play a part in the activations produced by the above-mentioned complexes, led to a study of proteins containing fixed SH groups, first described by Hopkins and Dixon [1922]. Proteins of this type, which occur in all plant and animal tissues, contain SH groups which are resistant to mild oxidation. Furthermore, the SH groups of these water-insoluble proteins cannot be removed by washing with water or dialysis through animal membranes, which indicates that these groups are an essential part of the structure of the protein molecule. Aqueous solutions of such proteins cannot be obtained by direct extraction. However, if the organs are freed from blood, minced and thoroughly washed with water, most of the SH proteins may then be extracted with excess secondary phosphate. It was found that these dissolved proteins are not attacked enzymically by activated papain. The extracts thus prepared give a positive nitroprusside test, which is intensified by addition of KCN, indicating the presence of fixed, reducible SS groups in addition to SH. The same conclusion was reached by Hopkins and Dixon [1922].

The following series of experiments was carried out to determine to what extent the addition of solutions of SH- and SS-proteins influences the activation of purified papain by vitamin C—Fe″, and whether these proteins may not be among the contaminating substances in commercial papain which cause the latter to respond differently to vitamin C—Fe″.

Preparation of fixed SH- and SS-proteins.

The organ, or preferably muscular tissue, of horse, ox, rabbit *etc.*, is freed from fat and nerve tissue and finely ground. 100 g. of the ground tissue are repeatedly kneaded with small portions of 0·25 % NaCl solution until the mass becomes greyish white. Most of the NaCl is removed by kneading with 2 volumes of distilled water. The mass is filtered with suction and the residue thoroughly kneaded with a small amount of $M/10$ Na_2HPO_4. More phosphate solution, up to a total volume of 150 ml., is then added. The mixture is shaken 2 or 3 hours and centrifuged. The supernatant fluid, which contains the desired proteins, may be preserved with toluene for several days in the ice-box.

Table III shows the effect of various activators on inactive papain in the presence and absence of fixed SH- and SS-proteins. The experimental details were identical with those described for Table I, except that in the cases indicated 7·0 ml. of the above-described fixed SH- and SS-protein solutions were added with the activator. The enzyme solution must not contain cathepsin. The time of incubation in each case was 20 hours.

It is evident from these results that purified inactive papain, which is not activated by vitamin C, pyruvic acid or succinic acid (or by their iron complexes), is nevertheless activated by these complexes in the presence of fixed SH- and SS-proteins. The magnitude of the activation is of the same order as that obtained with cysteine—Fe″. These findings demonstrate not only that the observed papain activation effects are dependent on the presence of accompanying substances, but also that these substances are to be sought for in the

Table III. *The effect of fixed SH- and SS-proteins on the activation phenomena of papain.*

Activator	Activity in ml. 0·05 N KOH	
	Without Fe″	With Fe″
None	0·00	0·00
Cysteine	4·80	5·00
Fixed SH- and SS-proteins	3·10	2·90
HCN	3·10	—
HCN + fixed SH- and SS-proteins	5·40	—
Vitamin C	0·00	0·05
Vitamin + fixed SH- and SS-proteins	3·10	4·90
Pyruvic acid	0·00	0·05
Pyruvic acid + fixed SH- and SS-proteins	3·10	4·20
Succinic acid	0·00	0·00
Succinic acid + fixed SH- and SS-proteins	3·10	4·70
Fixed SH- and SS-proteins + cysteine + papain (without gelatin)	0·08	0·05

class of proteins which contain fixed SS groups. The activator systems (vitamin C—Fe″, pyruvic acid—Fe″ and succinic acid—Fe″), in the presence of the papain complex, convert these SS-proteins into the SH-form, which is then able to activate the enzyme. It is also evident from Table III that this conversion of SS- into SH-protein (as a step in the activation process) requires the presence of bivalent iron. This reduction of SS to SH can also be followed by means of the nitroprusside reaction, as, for example, in the system HCN + fixed SS-protein. The part played by the bivalent iron in this process is not yet clear. Possibly the relationship is to be sought in an activation, by the iron, of dehydrogenases which are always found in the fixed SH- and SS-protein solutions prepared as described. The activation of papain by the system succinic acid—fixed SH- and SS-protein—Fe″ supports this view. It will be of interest to attempt to establish a connection between papain activation and the flavins (vitamin B_2). Our knowledge of this field has been greatly extended by the recent work of György *et al.* [1934] and Wagner-Jauregg *et al.* [1934].

It frequently happens that the protein solutions prepared as previously described contain mostly fixed SH-protein and very little of the SS-form. This can be seen from the high activation of inactive papain which they produce. In these cases also the nitroprusside reaction is not intensified by HCN or other activators. This latter finding in particular is evidence supporting the assumption that the conversion of fixed SS-protein into the SH-form by the various activators (vitamin C, pyruvic acid and succinic acid in presence of bivalent iron) is responsible for the activating effect of the latter. Further evidence for this is presented in the next section of this paper, in which a series of papain activation experiments was carried out with a solution of fixed SS-protein prepared by treatment of the tissue extract with alloxan.

Conversion of fixed SH-protein into the SS-form by means of alloxan.

It has been pointed out [Purr, 1935] that alloxan converts SH groups into SS groups without appreciably affecting the structure of the protein molecule. On the basis of this finding the following method was evolved for converting fixed SH-proteins into the SS-form.

100 ml. of a muscle extract prepared as previously described were treated for 5 hours at 37° with 0·7 g. of alloxan. A trace of toluene was then added and the excess alloxan removed by dialysis through an animal membrane (fish bladder), the desired protein being non-dialysable. At the end of the dialysis

the protein solution was divided into 2 parts, one of which was used immediately in the activation experiments. The other part was centrifuged to remove a precipitate which had formed during the dialysis, and the liquid was dried in a vacuum-desiccator over P_2O_5. The fine powder thus obtained contains mostly fixed SS-proteins (little SH) and was used directly.

A series of activation studies was carried out on reversibly inactivated papain using the dialysate and the dry powder obtained from alloxan-treated muscle extract, with and without HCN, vitamin C, pyruvic acid and succinic acid. Duplicate experiments were run with the addition of 20 mg. of $FeSO_4, 7H_2O$.

(*a*) *Before alloxan treatment.* Control experiments were run with a solution of fixed SH- and SS-proteins before alloxan treatment. These experiments were identical with those recorded in Table III.

(*b*) *Dialysate after alloxan treatment.* These experiments were identical with those described in (*a*) except that 10 ml. portions of the alloxan-treated dialysate were used instead of the untreated solution of fixed SH and SS groups.

(*c*) *Dry powder from alloxan-treated dialysate.* These experiments were identical with those described in (*a*) except that 60 mg. of the dry powder obtained from the alloxan-treated dialysate were used instead of the untreated solution of fixed SH- and SS-proteins.

Table IV. *Effect of fixed SS-proteins on the activation phenomena of papain.*

	Activity before alloxan treatment		Activity after alloxan treatment			
			Dialysate		Dry powder	
Activator	Without Fe″	With Fe″	Without Fe″	With Fe″	Without Fe″	With Fe″
None	0·00	0·00	0·00	0·00	0·00	0·00
Cysteine	4·90	5·00	4·80	5·00	4·90	5·00
Fixed SH- and SS-proteins	3·80	3·80	2·50	2·50	0·70	0·80
HCN	3·10	—	3·10	—	3·10	—
HCN + fixed SH- and SS-proteins	5·30	—	4·70	—	·	—
Vitamin C	0·00	0·05	0·00	0·00	0·00	0·00
Vitamin C + fixed SH- and SS-proteins	3·80	4·30	2·40	4·80	0·70	2·90
Pyruvic acid	0·00	0·05	0·00	0·00	—	—
Pyruvic acid + fixed SH- and SS-proteins	3·70	4·20	2·50	2·50	0·70	0·70
Succinic acid	0·00	0·00	—	–	—	—
Succinic acid + fixed SH- and SS-proteins	3·65	4·10	2·40	2·30	0·65	0·50

The results, shown in Table IV, indicate that it is possible to convert fixed SH-protein into the SS-form. It is also clear that the activation of inactive papain, obtained with fixed SS-proteins in the presence of the vitamin C—Fe″ complex, is an indirect effect resulting from the formation of fixed SH groups. The vitamin C—Fe″ complex is able, in the presence of the papain complex, to convert fixed SS-protein into the corresponding SH-form. This follows from the fact that addition of vitamin C—Fe″ scarcely affects the papain activity obtained with a fresh, untreated solution of fixed SH- and SS-protein (containing much SH-protein and very little of the SS-form). The observed activation is due merely to SH groups present in the protein solution, since approximately the same degree of activation results in the absence of vitamin C—Fe″. When however a mixture of fixed SH- and SS-proteins (alloxan treatment) is used, the partial papain activation can be increased to full activation by addition of vitamin C—Fe″. This is true also when the latter is replaced by HCN. Full papain activation is obtained also with pyruvic acid—Fe″ and succinic acid—Fe″, provided the fresh (untreated) SH- and SS-protein solution is also added. However, for unknown reasons these two complexes lose their activating effects when alloxan-treated preparations of SH- and SS-proteins are used.

Distribution of fixed SH- and SS-proteins.

Fixed SH- and SS-proteins, which, on the basis of the above results, play a part in the activation of papain by vitamin C—Fe″, are widely distributed in plants and animal organs. As examples of animal tissue, ox muscle, rabbit muscle, rabbit liver and rat sarcoma (Philadelphia #1) were extracted by the previously described method for obtaining solutions of these proteins. The activating effects of these solutions (7 ml.) on inactive papain were then measured in the presence of iron or vitamin C—Fe″, the experimental details being identical with those previously described. As examples of the plant kingdom, barley meal and jack bean meal were studied. The quantities used were 100 mg. of meal, 0·2 ml. of inactive papain, 20 mg. of $FeSO_4, 7H_2O$ (in 1 ml. of water) and 7 mg. of vitamin C (in 1 ml. of water). The total volume was brought to 7 ml., and the mixture was then incubated for 30 minutes at 37° and p_H 7. The measurement of papain activity followed as usual. Similar experiments were carried out without the addition of vitamin.

Table V. *Distribution of fixed SH- and SS-proteins in plant and animal cells, and their relation to the vitamin C activation of papain.*

Activator	Activity without vitamin C (Fe″ present)	Activity with vitamin C (Fe″ present)	Time of incubation (hours)
None	0·00	0·00	20
Cysteine	5·00	—	20
Ox muscle	3·70	5·0	20
Rabbit muscle	3·10	4·80	20
Rabbit liver	3·80	4·90	20
Rat sarcoma	3·50	5·0	20
Barley meal	0·70	2·50	20
Barley meal and cysteine	5·00	—	20
Jack bean meal	1·00	2·10	20

Table V shows that fixed SH- and SS-proteins probably occur in most plant and animal cells. This is evident from the papain activations obtained in the presence or absence of vitamin C. As previously stated, the activation obtained with the protein solutions in the absence of vitamin is due only to the fixed SH-protein present. The increased activation which results on addition of vitamin C—Fe″ is due to the conversion of the SS-proteins into the SH-form by the vitamin C—Fe″ in the presence of the papain complex. HCN has a similar effect.

Experiments with SS-glutathione.

Hopkins and Dixon [1922] and Bernheim and Dixon [1928] found that fixed SH-proteins (bound to the cell residue) would reduce oxidised glutathione to the SH-form. It was of interest, therefore, to investigate the effects of SS-glutathione, fixed SH- and SS-protein suspension and vitamin C on papain activation.

A solution of oxidised glutathione was prepared by passing oxygen through a solution of 100 mg. of reduced glutathione in 10 ml. of water, in the presence of a trace of $FeSO_4, 7H_2O$. Twelve hours' oxygenation were usually sufficient to complete the reaction, the course of which was followed by means of the nitroprusside test, and by the iodimetric procedure of Woodward and Fry [1932].

To 10 mg. of oxidised glutathione in 1 ml. water were added 20 mg. of $FeSO_4, 7H_2O$ (in 1 ml. of H_2O) and 6 ml. of a 1:5 aqueous suspension of ox muscle, which had previously been minced and thoroughly washed with water. The mixture was incubated at p_H 7 and 37° for 1 hour, after which 0·2 ml. of inactive papain extract was added. The incubation was continued for an additional hour. The papain activity was then measured as previously described. Control experiments were carried out in a similar manner on oxidised glutathione alone and muscle suspension alone. In a second series, 10 mg. of oxidised glutathione were treated with 20 mg. of $FeSO_4, 7H_2O$, 7 mg. of vitamin C and 2 ml. of inactive papain, in a total volume of 6 ml. After 1 hour's incubation at p_H 7 and 37°, the papain activity was measured in the usual manner. A control was run without oxidised glutathione. The results are shown in Table VI. Time of incubation in all cases was 20 hours.

Table VI. *Relation of fixed SH-proteins and the vitamin C—Fe″ complex to glutathione metabolism.*

Activator	Activity (ml. 0·05N KOH)	
	Without Fe″	With Fe″
None	0·00	0·00
Cysteine	4·9	4·30
Oxidised glutathione	0·50	0·30
Ox muscle	3·5	3·40
Oxidised glutathione and ox muscle	4·6	4·90
Vitamin C	0·00	0·05
Vitamin C and oxidised glutathione	0·60	1·50

The reduction of SS-glutathione to the SH-form by fixed SH groups, which was first followed analytically by Hopkins [1926], has now been demonstrated enzymically by means of papain activation. Such a reduction process is probably related to intracellular proteolysis. A similar reduction of oxidised glutathione (or oxidised papain?) is obtained when oxidised glutathione and vitamin C—Fe″ are added to inactive papain. Since neither oxidised glutathione—Fe″ nor vitamin C—Fe″ is able to activate inactive papain, it appears likely, as in the case of fixed SS-proteins, that the vitamin C—Fe″ complex and the papain are jointly responsible for the reduction of the SS group. In the absence of papain no reduction can be observed. The activation of papain by vitamin C—Fe″ apparently depends on the presence of SS groups, either in the glutathione molecule or in fixed SS-protein.

Activation of cathepsin.

In these experiments, an inactive cathepsin preparation was used, which was made by acetone-ether extraction of minced rabbit liver [Purr, 1934]. The dry powder thus obtained was extracted for 12 hours with 10 parts of 90 % glycerol and then filtered. 5 ml. of the filtrate were kept for 30 minutes at p_H 7 and 30° with 6 ml. of a solution of fixed SH- and SS-proteins (prepared from ox muscle as previously described), 20 mg. of $FeSO_4, 7H_2O$ in 1 ml. of water and 7 mg. of vitamin C in 1 ml. of water. After incubation the enzymic activity was measured essentially as described for papain. 5 ml. of 8 % gelatin and 2 ml. of M sodium acetate buffer of p_H 4 were added. 10 ml. of the reaction mixture were analysed immediately for amino-nitrogen, and 10 ml. after 24 hours' incubation at 37° by the Van Slyke method, the results being expressed in ml. of 0·05N KOH. Controls were run in the absence of (*a*) vitamin C, (*b*) fixed SH- and SS-protein and (*c*) fixed SH- and SS-protein and vitamin C. A similar reaction was carried out using 20 mg. of pyruvic acid in place of the vitamin C.

Also the full activity was determined by keeping 5 ml. of cathepsin solution for ½ hour at p_H 7 and 30° with 20 mg. $FeSO_4, 7H_2O$ in 1 ml. of H_2O and 20 mg. of cysteine hydrochloride in 1 ml. of H_2O. The activity of the enzyme was measured in the usual way; time of action 24 hours.

Table VII. *The effect of fixed SH- and SS-proteins on the activation phenomena of cathepsin.*

Activators	Activity (ml. 0·05 *N* KOH) (with Fe″)
None	0·00
Cysteine	1·40
Vitamin C	0·6
Fixed SH- and SS-proteins	0·40
Vitamin C and fixed SH- and SS-proteins	1·50
Pyruvic acid	0·00
Pyruvic acid and fixed SH- and SS-proteins	0·25
Fixed SH- and SS-protein and gelatin (no cathepsin)	0·00
Fixed SH- and SS-protein and cysteine and cathepsin (no gelatin)	0·01

The results shown in Table VII indicate that, as far as they have been studied, the activation phenomena of cathepsin are quite similar to those of papain. Only in the case of pyruvic acid did the results differ. Here it was found that the system fixed SH- and SS-protein—pyruvic acid—Fe″ does not increase catheptic activity.

SUMMARY.

1. It has been shown that the activation of papain by the vitamin C—Fe″ complex is dependent upon the presence of contaminating substances. Among the latter were found fixed SS-proteins and SS-glutathione.

2. Evidence is produced to show that the activation of papain and cathepsin by the vitamin C—Fe″ complex or by HCN is an indirect effect. The real activators are sulphydryl compounds, which are formed from the SS compounds which accompany the enzyme complex (fixed SS-proteins and SS-glutathione), by the action of the activator system mentioned. In contrast to arginase activation, the activation of papain and cathepsin is probably produced only by sulphydryl compounds.

3. It is concluded that papain and cathepsin in the active state are fixed SH-proteins of definite constitution, while the reversibly inactivated forms of these enzymes appear to be SS-proteins.

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REFERENCES.

Bernheim and Dixon (1928). *Biochem. J.* **22**, 125.
György, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 241.
Hopkins (1926). *Skand. Arch. Physiol.* **43**, 33.
—— and Dixon (1922). *J. Biol. Chem.* **54**, 523.
Maschmann and Helmert (1934). *Z. physiol. Chem.* **224**, 56.
Purr (1933). *Biochem. J.* **27**, 1703.
—— (1934). *Biochem. J.* **29**, 5.
—— and Weil (1934). *Biochem. J.* **28**, 740.
Wagner-Jauregg, Rauen and Möller (1934). *Z. physiol. Chem.* **224**, 67.
Waldschmidt-Leitz, McDonald *et al.* (1933). *Z. physiol. Chem.* **219**, 115.
Woodward and Fry (1932). *J. Biol. Chem.* **97**, 465.

III. THE CHEMISTRY OF MOULD TISSUE.

VI. FACTORS INFLUENCING THE AMOUNT AND NATURE OF THE FAT PRODUCED BY *ASPERGILLUS FISCHERI*[1].

BY EDWARD ALBERT PRILL, PETER RUDOLF WENCK
AND WILLIAM HAROLD PETERSON.

From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.

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THE fat content of mould mycelium, as reported by various authors, ranges from 1 to 40 %, depending upon the species and upon the conditions of growth. Even within a single strain of a species certain characteristics of the fat, as well as its amount, have been reported to vary with the conditions of growth.

From what is known about the chemical composition of mould-fat it appears that glycerides together with free fatty acids constitute the major portion of it. In some cases the mould-fat contains a high percentage of free fatty acids [Pruess *et al.*, 1934]. Barber [1929] found that the nature of the fatty acids, both free and combined, produced by a *Penicillium* sp. remained practically unchanged whether this mould was grown on glucose, sucrose, xylose or glycerol. The more easily extracted fat of *A. sydowi*, exclusive of the phospholipins [Strong and Peterson, 1934] and that of *P. javanicum* [Ward and Jamieson, 1934] have both been shown to yield upon saponification glycerol and oleic, linoleic, palmitic, stearic and small amounts of *n*-tetracosanoic acids. The mould-fat contains variable percentages of phospholipins and sterol, the latter having been definitely shown to be ergosterol in the case of *A. fischeri* [Pruess *et al.*, 1932]. There also occurs a small amount of some other unsaponifiable material which has not yet been studied. A small amount of pigment is presumably also present, since the mould-fat is usually quite deeply coloured.

The mould-fat has been generally regarded as a reserve material [Perrier, 1905], although Kordes [1923], observing that the fat globules in the hyphae persisted even during inanition, believed that this fat should be considered as an "excretory" product. Belin [1926] demonstrated that the fat content of *A. niger* fell to a low value after prolonged inanition. He postulated that the fats of moulds and of other living organisms should be considered as consisting of an "élément constant" and an "élément variable." By the term "élément constant" he designated that minimum percentage of fatty material, assumed by him to be phospholipins, intimately associated with the protoplasm, which is indispensable for the life of the organism; and by the term "élément variable" he designated any additional amount of fat stored as a reserve material.

In the case of animal tissues, three possibilities have been postulated for the function of the phospholipins [Sinclair, 1934]: (1) that they are intermediate

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products in fat metabolism; (2) that they act as oxygen transport agents within the cells; and (3) that they, and possibly also the cholesterol, are concerned in some more strictly physicochemical sense in the structural make-up and activity of the cell. It is possible that in the mould cells the phospholipins and also the sterol (ergosterol) might have functions similar to those postulated above.

The purpose of the present investigation was to determine the effects of the variations of certain factors on the amount and nature of the fat produced by *A. fischeri*, and also to determine any relationships which might exist among the percentages of total fat, phospholipins and sterol in the mycelium.

EXPERIMENTAL.

The mould was grown in 500 ml. pyrex Erlenmeyer flasks, six to eight flasks for each condition of growth, each flask containing 100 ml. of inorganic salt solution to which cerelose (commercial glucose) was added in the amount of 20 g. unless otherwise indicated. Except for certain indicated changes in the amount or nature of the nitrogen source in some of the experiments, the composition of the inorganic salt solution per flask was as follows:

NH_4NO_3	1·00 g.
KH_2PO_4	0·68 g.
$MgSO_4, 7H_2O$	0·50 g.
$FeCl_3, 6H_2O$	0·016 g.
$ZnSO_4, 7H_2O$	0·005 g.
Distilled water	100·0 ml.

To prevent the development of acidity, $CaCO_3$ was added to the media for all experiments except those concerned with the effects of acidity or alkalinity. Those components of the media which would react with each other if heated together, *e.g.* glucose and an alkaline substance, were sterilised separately.

Except for the comparison of the different cultures, in which case soil suspensions of these cultures were used as inocula, an aqueous suspension of the spores from a malt-agar slant of a single spore culture of *A. fischeri* was used as the inoculum in each series of experiments.

At the end of the growth period the mould was killed by autoclaving at 120° for 10 minutes. The mould pads were removed, washed with water, air dried at 37° for 2 days, weighed and ground to pass a 40-mesh sieve. The amounts of unutilised glucose remaining in the media were determined by the Shaffer and Hartmann [1920–21] method.

For the analyses 10 g. samples of the dry mould were weighed out, usually in duplicates if the amounts of material permitted. The samples were extracted with hot absolute alcohol in continuous extractors for at least 12 hours. It was found that this solvent extracted the fat from the mould more completely than did chloroform, ether or light petroleum. Moreover, it was also found that comparatively negligible amounts of additional fatty acids were obtainable upon more drastic treatment of the alcohol-extracted residue with alkali or acid. Since the alcohol also extracted some materials other than fat, the alcohol was distilled from the extracts, and the mixtures of fat and other extracted matter were dried in a vacuum-desiccator over $CaCl_2$ and then treated with hot chloroform, which dissolved only the fat. The chloroform-insoluble material was usually a light yellow water-insoluble powder, which comprised 10–30 % of the total alcoholic extract and 2–4 % of the mould pad. In some cases, which will be mentioned later, the chloroform-insoluble material was of a different nature, *i.e.* it was gummy and appeared to contain a large amount of carbohydrate. In these latter cases this fraction comprised the major portions of the alcoholic extracts and in an extreme case it formed 36 % of the mould pad.

The chloroform solution of the fat was in each case made up to a volume of 50 ml. For the determination of the amount of total fat a 20 ml. portion of this solution was evaporated in a tared 50 ml. Erlenmeyer flask, dried in a vacuum-oven at 60° for 2 hours and weighed. An aliquot of this same portion of fat was used for the determination of phosphorus by the Fiske and Subbarow [1925] colorimetric method after ignition with $Mg(NO_3)_2$. In some cases another aliquot of this fat was used for the nitrogen determination [Chiles, 1928].

The $CHCl_3$ was completely removed by distillation from the other 30 ml. portion of the solution and the fat was saponified by boiling for 1½ hours with 50 ml. of 5 % alcoholic KOH. After removal of the greater portion of the alcohol by distillation and addition of 50 ml. of cold water, the unsaponifiable matter was extracted by shaking with three successive 50 ml. portions of ether, the liquids being allowed to stand in the separating funnel until the two layers were distinct and free from emulsions. The combined ether solution of the unsaponifiable matter was washed once with 1 % KOH and twice with water, these washings being added to the aqueous soap solution. The aqueous soap solution was made strongly acid with HCl, and the fatty acids were extracted with ether. After washing this extract with water, distilling off the ether and drying the crude fatty acids in a vacuum-desiccator over $CaCl_2$, light petroleum was used to redissolve the true fatty acids. The small amount of more deeply coloured residue insoluble in light petroleum amounted to 3 % or less of the original fat. After distilling off the solvents and transferring to tared 50 ml. Erlenmeyer flasks, both the unsaponifiable matter and the fatty acids were dried in a vacuum-oven at 60° for 2 hours. This method of drying did not affect the iodine numbers of the fatty acids. The weight obtained for the fatty acids corresponds to that of the non-volatile fatty acids. These were in all cases light yellow in colour and appeared to be solid up to about 30°.

Aliquots of the fatty acids were taken for the determination of the iodine numbers and neutral equivalents. All the iodine numbers were determined by the Rosenmund and Kuhnhenn [1923] method, which, while reported to give somewhat lower values than the Wijs method, is less affected by experimental conditions and gives values which are consistent amongst themselves [Barbour, 1934; Yasuda, 1931]. The neutral equivalents, as determined by titration of an alcoholic solution of the fatty acids with $N/10$ NaOH to the phenolphthalein end-point, were all around 280, with maximum deviations of 5. Because these deviations are not much greater than the experimental error, the individual neutral equivalents are not included in the Tables.

The amount of sterol in the unsaponifiable matter was determined by a colorimetric method based on the Liebermann-Burchard reaction. This method will be described in a later publication.

Comparison of cultures.

Nine different cultures of *A. fischeri* Wehmer, eight of which were recently developed from single spores [Greene, 1933], were grown at 30° on a medium containing 10 % glucose. A series of old soil suspensions (A) and another series of more recently prepared soil suspensions (B) of these cultures were used as inocula. Practically all the glucose was utilised at the end of the 10-day growth period. The yields of mould pad per g. of glucose utilised varied only slightly from the average value of 0·27 g. In Table I the single spore cultures (S.S.C.) and the parent stock culture are arranged in the approximate order of the decreasing fat contents of the mycelia which they produced.

Table I. *Comparison of cultures.*

			Percentages of fat					Iodine no. of fatty acids
S.S.C. No.	Series	Fat %	Fatty acids	Unsap. matter	Sterol	P	N	
13	A	20·5	70·3	5·9	3·6	0·51	—	81
	B	16·1	70·7	8·5	4·7	0·52	0·38	79
7	A	15·2	68·5	8·9	4·6	0·53	—	79
	B	14·8	70·0	9·6	4·8	0·47	0·38	79
10	A	14·9	58·0	11·3	6·5	0·59	—	87
	B	15·1	62·2	11·5	6·4	0·58	0·43	84
1	A	14·8	65·3	9·1	5·7	0·47	0·30	77
	B	14·4	66·8	11·1	5·3	0·42	0·32	73
12	A	13·5	63·6	9·5	5·3	0·35	—	83
	B	14·5	67·0	9·9	5·5	0·52	0·43	83
2	A	12·7	64·4	10·8	5·8	0·53	0·22	79
	B	11·0	65·9	11·9	5·1	0·40	0·33	76
9	A	12·5	60·4	12·7	6·3	0·58	—	80
	B	11·5	63·2	13·0	7·6	0·50	0·42	78
20	A	11·2	55·0	10·2	4·2	0·49	0·24	89
	B	10·5	55·2	12·2	5·3	0·51	0·32	89
Stock	A	10·7	54·6	12·0	6·4	0·63	—	86
	B	11·2	58·1	9·2	4·7	0·45	0·28	89

It is seen that the variations in the amount and nature of the fats produced by the different cultures were quite appreciable and were fairly parallel in the two series. The pads produced by S.S.C. No. 13 were the highest in fat content, while those produced by S.S.C. No. 20 and by the stock culture were the lowest. In those cases where the fat content of the mould was low the fatty acids made up comparatively low percentages of the fat (about 55 % in the extreme cases) and the unsaponifiable matter tended to make up increased percentages; but the sum of these two components left considerable percentages of the fat unaccounted for, which might have been due to the presence of some other water-soluble components. The iodine numbers of the fatty acids produced by the different cultures showed fairly regular variations in both series and were highest in the cases of S.S.C. No. 20, S.S.C. No. 10 and the stock culture.

For the subsequent experiments the culture designated as S.S.C. No. 13 was used exclusively. It was believed that by using an inoculum as homogeneous as possible, *i.e.* all its components having the same characteristics, the observed variations in the amount and nature of the fat would be a true picture of the effects due to the variations in the conditions of growth; whereas, by using an inoculum, such as the stock culture, consisting of a mixture of components having different characteristics, the observed variations in the fat would be due partly to the altered proportions in which the different components would co-exist under the changed conditions of growth.

Effect of initial concentration of glucose.

The data for this series are summarised in Table II. The initial concentrations of glucose were varied by adding from 1 to 70 g. of cerelose to 100 ml. of the salt solution. The progressively increasing initial concentrations of glucose in

Table II. *Effect of initial concentration of glucose.*

Glucose added to 100 ml. of sol. g.	Glucose utilised g.	Period of incubation days	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat				Iodine no. of fatty acids
					Fatty acid	Unsap. matter	Sterol	P	
1·0	0·84	4	0·38	10·4	51·0	16·2	5·4	2·30	113
3·0	2·55	5	0·37	11·8	62·4	9·0	4·3	1·21	100
5·0	4·65	7	0·34	10·8	67·5	10·9	6·7	0·76	94
10·0	9·5	8	0·33	13·1	75·0	9·0	6·4	0·66	80
15·0	14·4	10	0·32	15·6	76·3	7·1	4·5	0·66	84
20·0	19·2	12	0·29	18·0	85·0	6·5	4·5	0·40	78
30·0	29·0	14	0·28	23·3	83·3	4·9	3·5	0·30	77
40·0	39·4	16	0·26	28·1	84·5	3·8	2·7	0·23	76
55·0	49·5	22	0·26	33·3	85·7	3·8	2·7	0·13	75
70·0	68·6	28	0·23	36·0	85·8	3·2	2·8	0·15	73

the medium caused certain quite regularly progressing variations in the amount and nature of the fat produced by the mould. The limited amount of nitrogen source compared with the large amount of carbon source in the media high in glucose was doubtless an important factor in producing some of these changes.

The fat content of the mould increased quite distinctly, which is in accord with previous reports [Belin, 1926]. In the case of yeasts also a high fat content is reported to be favoured by a high concentration of fermentable sugar and a low concentration of nitrogen in the medium [Smedley-MacLean, 1922]. Although the yields of mycelium per g. of glucose utilised decreased from 0·38 to 0·23 g., the yields of fat per g. of glucose utilised still showed an increase from 0·0395 to 0·083 g. These changes can be correlated because the conversion of carbohydrate into fat requires energy, hence greater portions of the glucose would have to be more or less completely oxidised in order to furnish the necessary energy for the synthesis of the greater amounts of fat. The "efficiency of the conversion of energy" [Terroine and Bonnet, 1927], which is equivalent to the value of the ratio,

energy content of the mould pad/decrease in energy content of the medium,

would doubtless have shown a much smaller decrease than did the yields in terms of the weight of mould pad per g. of glucose utilised.

The fatty acids obtained after saponification comprised increasing percentages of the original fat, while the unsaponifiable matter comprised decreasing percentages. Also the sum of these two components accounted for the fat more completely when the fat content was high. These facts, together with the decreasing percentages of phosphorus in the fat, indicate that the increment of fat consisted almost entirely of glycerides.

Based upon the mycelium, the percentages of unsaponifiable matter varied slightly around an average value of 1·2 %. The sterol usually comprised from 65 to 75 % of the unsaponifiable matter, except in the first two members of the series where the percentages were lower. Based upon the mycelium, the percentages of lipin-phosphorus were unusually high for the first two members (0·23 and 0·14) and decreased through the series, down to 0·05. Except for the first two members, the ratio of lipin-phosphorus to fat-free mycelium tended to remain fairly constant in this series.

The iodine numbers of the fatty acids decreased. The unusually high iodine numbers for the first three members of the series might be due to the more unsaturated character of the mould-phospholipins, which in these cases appear to make up appreciable percentages of the fat.

Effect of ammonium nitrate concentration.

The data for this series of experiments are summarised in Table III. The mould was allowed to grow at 30° for 10 days.

Table III. *Effect of ammonium nitrate concentration.*

NH_4NO_3 %	Glucose utilised %	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat					Iodine no. of fatty acids
				Fatty acids	Unsap. matter	Sterol	P	N	
0·2	44	0·45	22·3	81·0	3·6	2·2	0·23	0·18	90
0·5	68	0·37	20·8	78·8	3·9	2·6	0·27	0·26	91
1·0	79	0·33	17·9	79·5	5·6	3·4	0·34	0·39	88
2·0	92	0·35	15·8	81·2	6·7	3·3	0·22	0·37	84
5·0	73	0·25	15·2	78·8	6·7	3·4	0·16	0·33	83
10·0	47	0·20	14·1	80·0	5·0	Traces	0·23	0·45	83

It is seen that the utilisation of the glucose was most complete in the medium containing 2 % NH_4NO_3. It should also be mentioned that the chloroform-insoluble portions of the alcoholic extracts (not shown in Table III) were the greatest in those cases where the mould had been grown on a medium having either an extremely high or extremely low concentration of NH_4NO_3 and where the utilisation of the glucose had been least complete. In these cases this fraction was gummy and appeared to contain a large amount of carbohydrate. This fraction made up 28 % of the mould pad produced on the 10 % NH_4NO_3 medium.

With the increasing initial NH_4NO_3 concentration of the medium the yields of mould pad per g. of glucose utilised decreased, the fat content of the mycelium decreased, and certain characteristics of the fat changed progressively. The unsaponifiable matter formed an increasing percentage of the original fat while the fatty acids made up a fairly constant percentage (about 80). The iodine numbers of the fatty acids decreased.

The percentages of sterol and of lipin-phosphorus were highest in the mycelium produced on the usual 1 % NH_4NO_3 medium, both on the basis of the mycelium and on the basis of the fat.

The mould pads produced on the 10 % NH_4NO_3 medium, and to a lesser extent those produced on the 5 % NH_4NO_3 medium, were slimy and lacked turgidity. The abnormal growth is probably reflected in the very low sterol content produced on the 10 % NH_4NO_3 medium and in the unusually high values of the ratio, lipin-N/lipin-P, for the mould grown on the higher NH_4NO_3 concentrations.

Effect of acidity or alkalinity.

Ammonium salts of mineral acids have a tendency to produce acidity in the medium because of the liberation of mineral acid when the nitrogen of the ammonium radical is utilised. In the case of NH_4NO_3 this does not necessarily apply, because the mould can utilise both ammonium- and nitrate-nitrogen but not necessarily at the same rate. Likewise, the use of the nitrates of the alkali metals would be expected to decrease the acidity or produce alkalinity in the medium.

To determine the effects of the acidity or alkalinity which is produced in the medium by virtue of the nitrogen source, the mould was grown on media in which the NH_4NO_3 was in some cases replaced by equivalent amounts of various other nitrogen sources. Parallel experiments in which either $CaCO_3$ or potassium acetate was added to some of these media were also performed. The mould was in all cases allowed to grow at 30° for 12 days. The data for these experiments are summarised in Table IV.

Table IV. *Effect of acidity or alkalinity developed in the media by virtue of the nitrogen source.*

					Percentages of fat				
N source	Neutralising agent per flask g.	Glucose utilised %	Mycelium per g. glucose utilised g.	Fat %	Fatty acids	Unsap. matter	Sterol	P	Iodine no. of fatty acids
NH_4Cl	None	36	0·16	9·0	61·7	11·2	7·7	0·08	100
	$CaCO_3$	51	0·32	23·2	78·5	3·8	3·3	0·26	75
$(NH_4)_2SO_4$	None	47	0·075	10·3	59·0	14·8	7·5	0·14	98
	1 g. KOAc	84	0·20	11·6	67·0	9·5	7·7	0·18	93
	$CaCO_3$	66	0·34	20·3	76·0	5·8	3·9	0·24	82
NH_4NO_3	None	84	0·28	17·3	70·0	6·4	4·9	0·53	75
	$CaCO_3$	84	0·30	23·1	66·6	4·7	4·0	0·39	75
	0·5 g. KOAc	99	0·27	21·2	79·0	7·1	5·9	0·52	79
	1 g. KOAc	99	0·28	22·2	80·0	6·3	5·1	0·46	84
	2 g. KOAc	99	0·25	26·1	82·0	5·2	3·9	0·25	84
$NaNO_3$	None	88	0·24	25·7	78·0	5·2	3·4	0·33	82
$Ca(NO_3)_2$	None	55	0·39	16·8	69·5	6·2	3·1	0·27	58
Urea	None	98	0·26	21·7	69·3	10·4	4·8	0·63	81
	$CaCO_3$	22	0·26	14·3	86·5	5·3	3·4	0·31	68

The growth of the mould on the unneutralised $(NH_4)_2SO_4$ and NH_4Cl media apparently produced such a high degree of acidity that further growth was inhibited. The glucose was very incompletely utilised and the yields of mycelium per g. of glucose utilised were very low. The alcoholic extracts contained large amounts of chloroform-insoluble material similar to that previously described, and in the case of the mycelium produced on the NH_4Cl medium this amounted to 36 % of the mould pad. The mould pads produced on these media had low fat contents. Based on the mycelium, the percentages of sterol and unsaponifiable matter were as high as those observed in other cases: hence, these components amounted to appreciable percentages of the fat. The percentages of lipin-phosphorus were unusually low. The iodine numbers of the fatty acids were comparatively high.

The addition of $CaCO_3$ to these two media caused the production of good yields of mould pad per g. of glucose utilised and high fat contents of the mycelia. The addition of a small amount of potassium acetate partially neutralised the H_2SO_4 which was liberated during growth. The potassium acetate acts as a neutralising agent because of its own slightly alkaline reaction and probably also because of the production of the more alkaline $KHCO_3$ upon utilisation of the acetate radical as an additional carbon source. In this case the utilisation of the glucose was more complete, the yield of mould pad was fair, but the fat content was only slightly increased. The nature of this fat was intermediate between those of the fats produced by the mould on the $(NH_4)_2SO_4$ media with and without $CaCO_3$.

The growth of the mould on the unneutralised NH_4NO_3 medium was fairly normal; however, the addition of $CaCO_3$ or increasing amounts of potassium acetate caused an increase in fat content of the mycelium.

Urea, $NaNO_3$ and $Ca(NO_3)_2$ also served as suitable nitrogen sources and favoured the production of mycelia with high fat contents. The mould pads produced on the $Ca(NO_3)_2$ medium contained an abnormally high percentage of mineral matter (16 % ash). If corrections were made for the excessive mineral content of these pads (5 % is a more usual ash content), the fat content would appear as a higher percentage than that indicated in the table. The addition of $CaCO_3$ to the urea media proved unfavourable to growth, probably because of the development of excessive alkalinity. The iodine numbers of the fatty acids in the two last-mentioned cases were unusually low.

For the investigation of the effect of the initial p_H of the medium, the usual medium was modified by the substitution of an equivalent amount of urea in place of the NH_4NO_3, and H_2SO_4 or KOH was added to adjust the initial p_H to the values indicated in Table V. The mould was allowed to grow at 37° for 12 days.

Table V. *Effect of initial* p_H.

N reagent added per flask ml.	Initial p_H	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	N	Iodine no. of fatty acids
H_2SO_4 2	2	0·200	19·2	64·5	15·7	10·3	0·66	0·24	85
H_2SO_4 0·5	3	0·217	17·0	66·5	14·6	11·3	0·63	0·37	87
0	4·6	0·223	19·5	59·0	12·9	8·6	0·66	0·46	88
KOH 2	6	0·275	23·9	78·5	6·7	4·6	0·46	0·23	89
KOH 6	8	0·270	37·0	76·5	2·9	1·8	0·23	0·11	77

Probably because of the hydrolysis of urea to $(NH_4)_2CO_3$, either by mould enzymes or by ordinary chemical reactions, and because of the low buffer capacities of the media, a final p_H of about 7·5 was observed in all media except the one which had an initial p_H of 8. The growth of the mould on this last medium was unique in that the p_H decreased from 8 to 7, only 67 % of the glucose was utilised (whereas all the glucose was utilised in the other media), and the mycelium had a marked tendency to pile up against the walls of the flasks. This mycelium contained an unusually high percentage (37) of fat. Also, the iodine number of the fatty acids was lower in this case than in the others.

The addition of H_2SO_4 to the urea medium caused a slight decrease in the yields of mycelium, hardly any changes in the percentages of fat or of lipin-phosphorus, but a marked increase in the percentages of sterol and total unsaponifiable matter. The addition of KOH to the urea medium caused an increase in the yields of mycelium and an increase in the percentages of fat, but a decrease in the percentages of sterol, total unsaponifiable matter and lipin-phosphorus, both on the basis of the mycelium and on the basis of the fat.

Pontillon [1930; 1932–33] made a study of the effects of various nitrogen sources and the concomitant changes in the degree of acidity of the media upon the amount and nature of the fatty constituents of *A. niger*. It is difficult to correlate his results and ours; however, it appears that, in regard to the nature of the fat produced, *A. fischeri*, while showing considerable variations, does not tend to exhibit some of the rather extreme variations which, as his data imply, occur in the case of *A. niger*.

Effect of temperature.

The data for these experiments are summarised in Table VI. The mould was allowed to grow at the indicated temperatures until all the glucose was utilised.

Table VI. *Effect of temperature.*

Temp. ° C.	Period of incubation days	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat					Iodine no. of fatty acids
				Fatty acid	Unsap. matter	Sterol	P	N	
20	16	0·331	24·4	79·0	5·9	3·4	0·46	0·20	93
30	12	0·305	25·7	81·5	5·8	4·1	0·39	0·20	82
37	12	0·273	20·0	80·0	8·5	6·8	0·41	0·22	88

It appears that a temperature of 37° is somewhat less favourable for fat production than are the lower temperatures. The percentage of sterol (based on the mycelium) increased greatly while that of the lipin-phosphorus decreased slightly as the temperature of growth was increased.

It has been reported [Pearson and Raper, 1927; Terroine *et al.*, 1927] that in the case of two moulds, *A. niger* and *Rh. nigricans*, and also in the case of the timothy bacillus, the iodine numbers of the fatty acids obtained from these organisms decreased as the temperature at which they were grown increased. Our data do not lend much support to the supposition that this tendency is a general one; for although the iodine number of the fatty acids produced by the mould at 20° was somewhat higher, those of the fatty acids obtained from the mould grown at 30° and 37° were in an order the reverse of that which would have been expected.

Effect of increased aeration.

The data for this experiment are summarised in Table VII. This experiment was performed twice, (A) with the usual 500 ml. Erlenmeyer flasks, and (B) with 3 l. Currie flasks containing 750 ml. of medium. The medium contained 15 % glucose in both cases. For increased aeration a continuous current of sterile air was introduced just above the surface of the pads. The flasks containing the controls were plugged with cotton wool in the usual manner. The mould was in all cases allowed to grow at 25° for 10 days.

Table VII. *Effect of increased aeration.*

		Fat %	Percentages of fat					Iodine no. of fatty acids
			Fatty acid	Unsap. matter	Sterol	P	N	
A	Aerated	23·8	79·0	3·8	2·2	0·24	0·12	83
	Control	24·6	75·5	4·3	2·3	0·26	0·10	83
B	Aerated	27·0	79·0	3·5	2·4	0·21	0·09	82
	Control	29·8	75·5	3·4	2·0	0·23	0·11	79

With increased aeration the growth of the mould was more rapid and the glucose was practically all utilised at the end of the 10-day period. In the controls there were considerable amounts of unutilised glucose, but the yields of mycelial pads per unit of glucose utilised did not differ greatly from those produced with increased aeration. No changes in the amount or nature of fat that can be considered significant were found to be produced by increased aeration.

Effect of inanition.

The mould for this series of experiments was grown at 30°. The first sample of six flasks was taken for analysis after 10 days' growth at which time 77 % of the glucose had been utilised. The other pads were allowed to remain on the medium for longer periods of time. The data for this series are summarised in Table VIII and the changes in the amounts of some of the mycelial constituents per flask are shown graphically in Fig. 1.

Table VIII. *Effect of inanition.*

Period of incubation days	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	Iodine no. of fatty acids
10	0·320	23·3	65·0	5·0	2·8	0·45	83
20	0·245	16·2	70·7	9·2	5·6	0·57	82
30	0·212	13·9	69·6	12·4	8·6	0·41	81
40	0·177	11·6	65·0	18·5	12·4	0·11	79
50	0·165	11·5	60·6	19·1	13·5	0·10	78
68	0·074	12·1	65·5	14·7	9·8	Traces	82

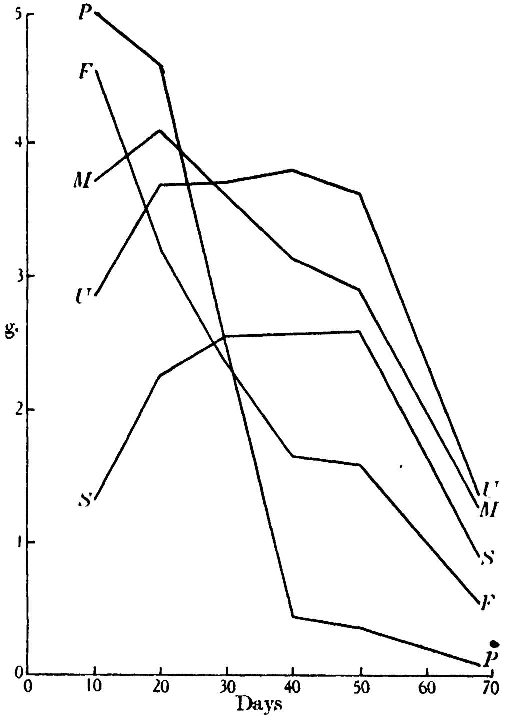

Fig. 1. Effect of inanition on some mycelial constituents, expressed as g. per 100 ml. of medium. M = mycelium *minus* fat; F = fat × 4; U = unsaponifiable matter × 50; S = sterol × 50; P = lipin-P × 1000.

Between the 10th and 20th days the absolute amount of fat began to decrease. The fat decreased more rapidly than did the sum of the other mycelial constituents until about the 40th day, after which both decreased at almost the same rate; consequently, the percentage of fat in the mould pad decreased from 23·3 to 11·6 and then remained fairly constant. Belin [1926] reported that on prolonged inanition the fat content of *A. niger* fell to a low final value corresponding to 1·37 % fatty acids on the basis of the final weight of the mycelium.

While a decrease in the absolute amount of fat was in progress, the amount of unsaponifiable matter, including the sterol, still increased slightly and then remained fairly constant for a long period before it also finally decreased. Until about the 50th day, the sterol and other unsaponifiable matter amounted to increasing percentages both of the fat and of the mould pad.

The fatty acids remained a fairly constant percentage of the fat. The iodine numbers of the fatty acids showed only a very slight tendency to decrease and the neutral equivalents varied only slightly around 280, just as was observed in the previous series of experiments. If these fatty acids are a mixture consisting of appreciable amounts of both saturated and more or less unsaturated individuals, the almost constant iodine number of this mixture would indicate that the unsaturated fatty acids are not preferentially utilised. It has also been reported [Pearson and Raper, 1927; Terroine and Belin, 1927] that the iodine number of the fatty acids obtained from *A. niger* is not greatly influenced by the period of incubation of the mould.

The amount of lipin-phosphorus decreased very rapidly between the 20th and 40th days. In view of the hypothesis that the phospholipins serve as intermediates in fat metabolism, it might be assumed that the phospholipin would be utilised more readily than the other fatty constituents. However, it is also possible that the observed decrease in lipin-phosphorus might have been due to an enzymic cleavage of the phosphate linkage rather than a complete utilisation of the phospholipin molecules.

DISCUSSION.

In the series of experiments dealing with the initial concentration of glucose, the yields of mycelium and of fat per g. of glucose utilised varied in opposite directions, and this was partly explained by the energy required for the conversion of carbohydrate into fat. In the other series studied the variations in the yields of both mycelium and fat were in the same direction, which indicates that in these cases the effect due to the above-mentioned principle was completely overshadowed by the effects due to other factors.

The percentages of sterol and other unsaponifiable matter, when based on the mycelium, were usually less variable than were the percentages of total fat: consequently, these components generally made up comparatively greater percentages of the fat in those cases where the fat contents of the mycelia were low. Also the undetermined water-soluble components left unaccounted for by the sum of the percentages of fatty acids and unsaponifiable matter amounted to comparatively greater percentages of the fat in these cases.

The amount of lipin-phosphorus was taken as an approximate measure of the amount of phospholipins. In those cases where the lipin-nitrogen was also determined, except for a few apparently abnormal cases, the value of the ratio N/P usually indicated between one and two atoms of nitrogen to one of phosphorus.

At a given temperature of growth and with media that did not become strongly acidic, the percentages of phospholipin (based on the mycelium) and of total fat tended to vary in opposite directions as the initial concentration of glucose, the concentration of NH_4NO_3 (up to 1 %) or the initial p_H was varied.

With a given initial concentration of glucose in the media, the percentages of sterol (based on the mycelium) and of total fat tended to vary in opposite directions as the degree of acidity, the concentration of NH_4NO_3 (up to 1 %) or the temperature of growth was varied.

It was observed that within a certain limited range of growth conditions—a temperature of 25° to 30°, a fairly neutral medium (presence of $CaCO_3$) and an initial concentration of glucose between 5 and 30 %—the values of the ratio, sterol/lipin-P, were usually within the range of 9 to 11. Under most other conditions of growth the value of this ratio was higher. If the free rather than the total sterol had been determined, an even more direct relationship might have been obtained. It has been suggested [Sinclair, 1934] that in animal tissues the sterol and phospholipin might be concerned in the regulation of some physicochemical property of the cells, and it has also been supposed that these two substances have antagonistic actions. Parallelisms between the percentages of cholesterol and phospholipins in different healthy organs of animals have been reported by numerous authors. In mould grown under a limited range of conditions there appears to be a parallelism between the percentages of sterol and phospholipin. In the mould grown under more extreme conditions different amounts and proportions of these two substances might be necessary for the adjustment of the mould cells to the external conditions.

The neutral equivalents of the fatty acids were in all cases very near to 280 (maximum deviation of 5). Although the neutral equivalent of a mixture of fatty acids is usually considered as the effective average molecular weight of the components, the almost constant value observed for the neutral equivalents of the fatty acids produced by *A. fischeri* under such diverse conditions suggests that there was a very great preponderance of those fatty acids which contain 18 carbon atoms.

SUMMARY.

1. Several single spore cultures of *A. fischeri* were compared in regard to the amount and nature of the fats produced in the mycelia. Appreciable differences were noted.

2. A study was made of the effects of the initial concentration of glucose, concentration of NH_4NO_3, acidity, alkalinity, temperature, increased aeration and period of incubation upon the amount and nature of the fat produced by a single spore culture of *A. fischeri*.

3. The production of mycelia with high fat contents was favoured by neutral or slightly alkaline media, a high initial concentration of glucose and a low concentration of NH_4NO_3.

4. The production of increased percentages of sterol in the mycelium was favoured by a fairly high initial concentration of glucose, 1 % NH_4NO_3 or an initially acid medium containing an equivalent amount of urea, a higher temperature (37°) and a long period of incubation.

5. The production of increased percentages of phospholipin in the mycelium, as judged by the percentages of lipin-phosphorus, was favoured by a low initial concentration of glucose, 1 % NH_4NO_3, and an initially slightly acid medium.

6. During inanition the greater part of the fat (exclusive of unsaponifiable matter) was utilised by the mould.

7. The iodine numbers of the fatty acids obtained from the total fat of the mould were higher when the mould was grown on a low concentration of glucose, on a medium which became strongly acid or at a lower temperature.

8. The neutral equivalents of these fatty acids were in all cases very near to 280.

REFERENCES.

Barber (1929). *Biochem. J.* **23**, 1158.
Barbour (1934). *Oil and Soap*, **11**, 7.
Belin (1926). *Bull. Soc. Chim. Biol.* **8**, 1081, 1120.
Chiles (1928). *J. Amer. Chem. Soc.* **50**, 217.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Greene (1933). *Mycologia*, **25**, 117.
Kordes (1923). *Bot. Arch.* **3**, 282.
Pearson and Raper (1927). *Biochem. J.* **21**, 875.
Perrier (1905). *Compt. Rend. Acad. Sci.* **140**, 1052.
Pontillon (1930). *Compt. Rend. Acad. Sci.* **191**, 1148, 1367.
—— (1932–33). *Rev. Gen. Bot.* **44**, 417, 465, 526; **45**, 20.
Pruess, Eichinger and Peterson (1934). *Zentr. Bakt.* II Abt. **89**, 370.
—— Peterson and Fred (1932). *J. Biol. Chem.* **97**, 483.
Rosenmund and Kuhnhenn (1923). *Z. Nähr. Genussm.* **46**, 154.
Shaffer and Hartmann (1920–21). *J. Biol. Chem.* **45**, 370.
Sinclair (1934). *Physiol. Rev.* **14**, 351.
Smedley-MacLean (1922). *Biochem. J.* **17**, 720.
Strong and Peterson (1934). *J. Amer. Chem. Soc.* **56**, 952.
Terroine and Belin (1927). *Bull. Soc. Chim. Biol.* **9**, 12.
—— and Bonnet (1927). *Bull. Soc. Chim. Biol.* **9**, 588.
—— —— Kopp and Véchot (1927). *Bull. Soc. Chim. Biol.* **9**, 605.
Ward and Jamieson (1934). *J. Amer. Chem. Soc.* **56**, 973.
Yasuda (1931). *J. Biol. Chem.* **94**, 401.

IV. FLAVIN CONTENT OF ANIMAL TISSUES UNDER DIFFERENT CONDITIONS.

By ABRAM J. CHARITE and NICOLAY W. KHAUSTOV.

From the Laboratory of Animal Biochemistry, Academy of Sciences, Leningrad.

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A NUMBER of communications during recent years by Warburg and Christian [1932, 1, 2] show that besides the iron-containing enzyme of a haemin nature there exists another enzyme capable of transporting oxygen under aerobic conditions and of dehydrogenation under anaerobic conditions. The scheme of its action is as follows:

1. Leuco-enzyme + oxygen = yellow enzyme + hydrogen peroxide (under aerobic conditions).
2. Leuco-enzyme + methylene blue = yellow enzyme + leucomethylene blue (under anaerobic conditions).

The power of flavins to oxidise by means of dehydrogenation has also been confirmed by Wagner-Jauregg *et al.* [1934] and by Adler and Euler [1934]. Freed from its colloidal carrier the yellow oxidation enzyme is identical with the flavin discovered and described in the same year by Kuhn *et al.* [1933, 1, 2] and simultaneously by Ellinger and Koschara [1933]. These same authors have shown in their investigations that the flavins are quite similar to vitamin B_2 in their action on the organism, and this makes these compounds the more important. Notwithstanding the great biological significance of the flavins, there do not exist sufficiently precise methods for their determination in animal tissues and organs. György *et al.* [1934] have drawn up a table of the flavin content of tissues according to their degree of action as carriers of vitamin B_2. Euler and Adler [1934] have compared the intensities of the colour of aqueous extracts from different tissues with a standard scale of solutions of flavins in different concentrations. The data obtained by Euler agree well with the data obtained by György *et al.* These methods however do not supply absolute values of the flavin content, but give only approximations.

We believe the method of Warburg and Christian [1933] to give more exact determinations. These authors consider it possible to calculate the concentration of flavins by the intensity of the absorption spectra of methanol extracts. This method of determination is however hardly available for most laboratories as it requires complicated equipment; we have therefore modified it by substituting colorimetry for spectrophotometry.

Method of determination.

Our method of determination is as follows. A definite amount of the organ is powdered and repeatedly washed in water until the water remains clear. The tissue is then extracted with 10 parts of a mixture of 3 vols. methanol with 1 vol. water for 36–48 hours at 28–36°. The extract is centrifuged and the upper transparent layer, containing all the flavins of the tissue under examination, is decanted into a separating funnel and treated with an equal volume of chloroform. The other pigments associated with the flavins pass into the chloroform; treatment with chloroform also clarifies the methanol layer. The lower chloroform layer, usually uncoloured (if the flavins of the kidneys, heart and liver are being examined), is drawn off, and the methanol layer is used for the determinations. The yellow flavin solution in methanol, having a green fluorescence, is compared in a colorimeter with a standard solution of potassium chromate containing 0·1 mg. $KCrO_4$ in 1 ml. This solution corresponds in colour to 1 ml. of an extract containing 2·37γ of flavin as shown below. Colorimetric determinations are possible for two reasons: (1) methanol extracts of the liver, kidneys, heart, brain or lungs do not contain any other pigments, as has been demonstrated by the absorption spectra of extracts of these organs; (2) at these concentrations the solution behaves according to Beer's law that the intensity of the colour is proportional to the concentration of the substance. In Table I is given an example of a determination of the flavin in the cortical substance of the kidney. 9·4 g. of the tissues were treated as above described. The volume of the methanol extract after treatment with chloroform was 70 ml. The solution was yellow-green in colour. The absorption spectrum was determined in the usual manner according to Warburg [1933].

SPECTROPHOTOMETRIC DATA.

Table I. *Measurements of the absorption of light by a methanol extract of the cortical substance of the kidneys.*

λ $m\mu$	I_0	I_1	$\frac{I_0}{I_1}$	λ $m\mu$	I_0	I_1	$\frac{I_0}{I_1}$
510	27·5	25·5	1·08	450	3·6	1·95	1·85
500	21·5	18·2	1·18	448	2·95	1·55	1·91
490	15·7	11·9	1·32	445	2·8	1·5	1·86
430	12·4	8·3	1·47	440	2·1	1·2	1·75
470	8·5	5·2	1·64	430	1·127	0·7	1·61
460	5·9	3·3	1·78	420	0·7	0·5	1·5
455	3·5	1·85	1·88	415	0·35	0·25	1·4

As the table shows, maximum absorption occurs at a wave-length of 448$m\mu$ in the visible spectrum. This fully agrees with the data obtained by Warburg and Christian [1933] for lactic acid bacilli and yeast. The flavin concentration calculated by the formula of the above authors was 0·2406 mg. in the 70 ml. extract or 25·6γ per g. of kidney cortex.

The methanol extract was then measured by the colorimetric method. The standard solution, as has been mentioned above, contains 0·1 mg. $KCrO_4$ in 1 ml. Comparison showed that the intensity of the colour of 1 ml. of the extract corresponded to the colour of 0·145 mg. $KCrO_4$ in 1 ml. But 1 ml. of extract contains 0·2406/70 mg. or 3·44γ of flavin. Hence 0·1 mg. $KCrO_4$ in 1 ml. will correspond in colour to 3·44/1·45 or 2·37γ flavin in 1 ml. This method is suitable for making determinations of flavins in those tissues and organs where the aqueous methanol extract does not contain other pigments, colouring the solution yellow. The liver, kidney (cortical substance), heart and brain belong to such tissues. Other tissues such as the spleen and, especially, blood contain a number of other pigments which influence the colorimetric determinations. Spectrophotometric analysis of the extract of the blood of the white rat for example clearly demonstrates the presence of three maxima, probably corresponding to the haemins (419$m\mu$, 474$m\mu$ and 575$m\mu$). It is evident therefore that colorimetric determination of flavins in blood cannot be based on the yellow colour of aqueous methanol extracts.

Flavin contents of various organs.

Using this method, so convenient for its rapidity and precision, we determined to have the flavin contents of the organs and tissues of several animal species. In Table II the flavin contents of the organs and tissues of a cat and a rabbit are given.

Table II. *The flavin contents in γ per g. of tissues and organs of a rabbit and a cat.*

Species	Liver	Kidney	Kidney cortex	Kidney medulla	Heart	Lung	Brain	Muscle
Rabbit	7·17	14·5	—	—	6·15	9·90	3·07	—
Cat	24·5	6·56	25·2	0	7·82	—	—	—

Our data on the distribution of flavins in the tissues and organs agree with the data of Euler and Adler [1934].

Effect of avitaminoses-A and -C.

We then carried out a series of experiments to determine the effect of avitaminosis on the flavin content of the animal body. Abderhalden's earlier work [1921] on the influence of avitaminosis in lowering the respiration of tissues served as the starting point for our experiments. The flavins form an oxidation-reduction system existing in every cell. We therefore considered it interesting to establish how much avitaminoses-A and -C influence the flavin contents of the livers of calves, rats and guinea-pigs. Determinations of the flavin contents of the liver and kidneys only were made, since these organs are richest in flavins. Table III summarises the results of our experiments.

Table III. *The flavin content in γ per g. of liver and kidneys of animals suffering from avitaminoses.*

Species	Normal	Avitaminosis-A	Avitaminosis-C
Calf (liver)	35·6	28·4	—
Rat (liver)	—	16·9	—
,,	-	28·4	--
,,	30·0	27·5	—
Guinea-pig			
Liver	20·8	—	22·0
,,	13·7	--	19·9
,,	11·9	—	--
Kidney	15·6	—	14·9
,,	—	–	19·2

A small decrease in the flavin content appears to occur in the liver of animals suffering from avitaminosis-A; avitaminosis-C however does not give a clear result; an increase in the flavin content occurred in the liver and a decrease in the kidneys.

Effect of rich protein diet.

The work of Tcherkess [1934] indicates that the lesions of pellagra are often caused by a faulty balance of proteins in the diet and sometimes by excessive protein in the diet of experimental animals. A high content of protein in the food exercises also a large specific dynamic effect. Wishing to establish to what extent these phenomena react on the course of oxidation-reduction processes, we made a number of experiments to determine the influence of a rich protein diet on the flavin content of the liver of white rats. The rats were sub-divided into two groups of eight animals in each, a control and an experimental group. The control group received the usual laboratory diet while the experimental group was fed on a diet rich in protein. The diet of this experimental group consisted of:

60 parts caseinogen (treated with alcohol);
40 parts starch (rice);
10 parts dry yeast;
1 part irradiated yeast;
4·8 parts Mendel-Osborne salt mixture.

The rats were kept for 28 days on such a diet. Table IV gives the flavin content in the liver of the experimental and control rats.

Table IV. *The effect of a rich protein diet on the flavin content γ per g. of liver of white rats.*

No.	1	2	3	4	5	6	7	8
Control	—	19·3	18·2	20·7	13·2	21·0	21·5	19·6
Experimental	20·3	20·4	19·2	18·5	22·9	21·6	19·3	20·3

The quantity of flavins in the liver does not apparently change significantly under the influence of a rich protein diet.

SUMMARY.

1. A method of determining flavins in some tissues and organs is described.
2. Data of the flavin contents of the tissues and organs of a cat and a rabbit are given.
3. In avitaminosis-A there is observed a slight decrease in the flavin content of the livers of white rats and calves.
4. The influence of avitaminosis-C is not clear.
5. A rich protein diet also has no definite effect on the flavin content.

REFERENCES.

Abderhalden (1921). *Pflüger's Arch.* **174**, 102.
Adler and Euler (1934). *Z. physiol. Chem.* **225**, 41.
Ellinger and Koschara (1933). *Ber. deutsch. chem. Ges.* **66**, 315.
Euler and Adler (1934). *Z. physiol. Chem.* **223**, 105.
György, Kuhn and Wagner-Jauregg (1933). *Naturwissensch.* **21**, 496.
—— —— —— (1934). *Z. physiol. Chem.* **223**, 21.
Kuhn, György and Wagner-Jauregg (1933, 1). *Naturwissensch.* **21**, 560.
—— —— —— (1933, 2). *Ber. deutsch. chem. Ges.* **66**, 317.
Tcherkess (1934). Fifth Congress of Physiologists of U.S.S.R. (Russian).
Wagner-Jauregg, Rauen and Moller (1933). *Ber. deutsch. chem. Ges.* **66**, 1298.
—— —— —— (1934). *Z. physiol. Chem.* **224**, 67.
Warburg and Christian (1932, 1). *Naturwissensch.* **20**, 688, 980.
—— —— (1932, 2). *Bioch. Z.* **254**, 438.
—— —— (1933). *Bioch. Z.* **266**, 377.

V. THE SPREADING OF COMPLEX PROTEINS.

By EVERT GORTER, HANS VAN ORMONDT
AND THOMAS MARINUS MEIJER.

*From the Laboratory of the Children's Hospital of the
University of Leiden, Holland.*

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SPREADING of proteins can be obtained by bringing a small amount of concentrated aqueous solution of the protein on to the surface of a Langmuir-Adam tray. In our experiments 0·5 % protein solutions and a micro-pipette containing 0·005 ml. are used. The pipette is held horizontally, so that the point is close to the water surface, and the content is slowly blown out. If conditions are favourable for spreading, measurements can be made after a very short time (1–2 minutes).

In order to determine the area occupied by a protein under different pressures, a glass slide is moved towards the floating barrier until this barrier moves away, thus indicating a small rise of the surface pressure.

The floating barrier can now be brought back into equilibrium by torsion of a spring mounted on a balance. The amount of torsion is read on a scale, which is calibrated in dynes per cm. of the floating barrier. The area is measured for different pressures and by extrapolating from these numbers, the area for zero pressure can be found. This part of the technique is identical with that described by Adam [1930] in his studies of the spreading of different substances.

It has been possible to show [Gorter, 1926; Gorter and Grendel, 1926; 1928; 1929; Gorter and Seeder, 1932; Gorter *et al.*, 1932, 1934] that spreading of a protein in a very thin layer (6–7·5 Å. thick) occurs under different conditions. From the experiments of Hughes and Rideal [1932], who used a somewhat different technique, this same thickness can be calculated.

The measured spreading (extrapolated to zero pressure and calculated in sq.m./mg. of spreading substance) is plotted against the p_H of the solution in the tray on which the substance is spread.

The buffers used in the tray were, amongst others, $M/300$ solutions of acetate-acetic acid covering the range p_H 3·6–5·6.

Spreading ovalbumin at a range of these p_H values, a maximum is found at p_H 4·6 (the isoelectric point) and very low minima of spreading on both sides of the isoelectric point, provided the solutions contain very few and only univalent ions.

By increasing the amount of electrolytes in the tray, the minimum spreading on both sides of the isoelectric point can be made to increase.

Multivalent negative ions appear to have a great influence on the minimum on the acid side (p_H 3·0), multivalent positive ions on that on the alkaline side (p_H 6·0) of the isoelectric point. Large amounts of univalent ions (± 40 millimol per litre) small amounts of bivalent ions (± 1 millimol per litre) or very small amounts of trivalent ions (± 1/40 millimol per litre) all produce similar effects: if they are positive they change the minimum spreading on the alkaline side into a maximum, if they are negative, they cause the disappearance of the minimum on the acid side.

Not only inorganic ions but also organic ions like glutathione on the acid side, or spermine on the alkaline side, produce the same effect, when added to the solution in the Langmuir tray. Moreover, a difference is seen between the members of a lyotropic series. Thus Li has a smaller influence than K, and more is needed of Cl to produce the same effect than of CNS [Gorter, 1934].

The explanation of these phenomena is that a protein like ovalbumin does not spread well in an ionised state, whereas the undissociated protein and its non-ionised salts do spread. Addition of ions to the water in the tray favours the spreading by discharging the protein ion.

These results have been obtained with different proteins: ovalbumin, pepsin and trypsin have been most completely studied.

It is the purpose of this paper to give the results of investigations of differences in spreading on different solutions in the tray between a simple protein and a complex protein artificially prepared therefrom.

In these complex proteins basic or acidic substances are combined with acid or basic groups of the protein.

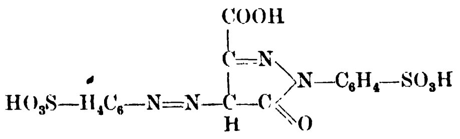
The spreading of complex proteins.

Many proteins are complex in so far as they contain a simple protein molecule to which a prosthetic group is attached, which is important for the behaviour of the molecule as a whole.

In order to elucidate the spreading of this group of proteins, we have prepared complex proteins by adding different substances to ovalbumin and to pepsin.

The first protein is very well suited for this study; it contains a great many free amino- and carboxyl groups and therefore the introduction of another molecule attached to these groups will be most likely to influence the mode of spreading at varying p_H.

Pepsin has a pronounced minimum on the alkaline side of the isoelectric point (p_H 2·85) and therefore is very appropriate for studying the influence of positive ions. Ovalbumin can be combined with tartrazin (I).



$$\begin{array}{l} \qquad\qquad\qquad\qquad\quad \mathrm{COOH} \\ \qquad\qquad\qquad\qquad\quad \mathrm{C{=}N} \\ \qquad\qquad\qquad\qquad\quad\ | \qquad \rangle \mathrm{N{-}C_6H_4{-}SO_3H} \\ \mathrm{HO_3S{-}H_4C_6{-}N{=}N{-}C{-}C} \\ \qquad\qquad\qquad\qquad\quad \mathrm{H} \qquad\ \ \mathrm{O} \end{array}$$

(I).

It is obvious that the latter, being a strong acid, must combine with the free NH_2 groups of the protein.

If we study the spreading of this complex protein, we see no change at the isoelectric point and on the alkaline side of this point, whereas the minimum on the acid side at p_H 2·7–3·0 disappears completely (Fig. 2).

Examination of the amount of tartrazin that must be added to an ovalbumin solution shows that 5·6mM tartrazin is sufficient for an ovalbumin solution containing 5 mg./ml., this being 1/7mM (mol. wt. 35,000). From these numbers we may calculate that ovalbumin contains 39 free NH_2 groups per molecule which combine with tartrazin to form a product showing maximum spreading at p_H 3·0.

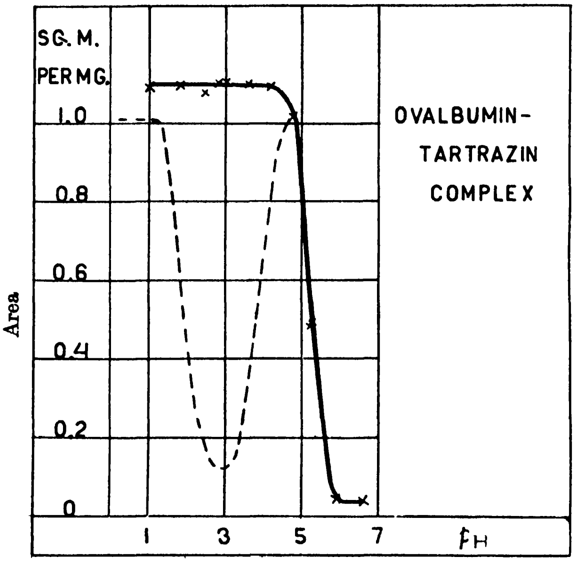


Fig. 1. The dotted curve shows the spreading of ovalbumin to which no tartrazin has been added. The continuous curve shows the area (in sq. m. per mg.) of the protein when spread on different solutions in the tray, the p_H of which is indicated on the abscissa. This ovalbumin-tartrazin complex contains 5 mg. ovalbumin per ml. and is 16*mM* with respect to tartrazin, an excess of which is present.

Measurements shown in this and other figures are made after 1–2 minutes unless otherwise stated.

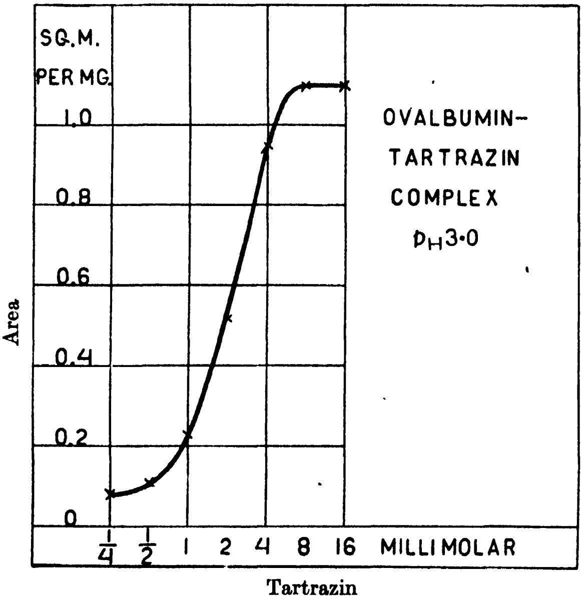


Fig. 2 Showing amount of tartrazin in millimols per litre that must be added to a 0·5 % ovalbumin solution in order to obtain spreading. The areas are indicated on the ordinate in sq. m. per mg.

If a less than 5·6*mM* solution of tartrazin is added to the same ovalbumin solution, some of the NH_2 groups only are bound and intermediate values for the spreading are obtained (Fig. 2).

The explanation must be that the protein now behaves as an acid and no longer as a zwitterion.

The reverse can be shown when a complex is prepared by adding to pepsin a substance like spermidine (II).

$$H_2N(CH_2)_3NH(CH_2)_4NH_2 \qquad \text{(II)}$$

This combines with the free COOH groups and now the curve of the spreading at varying p_H has the form shown in Fig. 3.

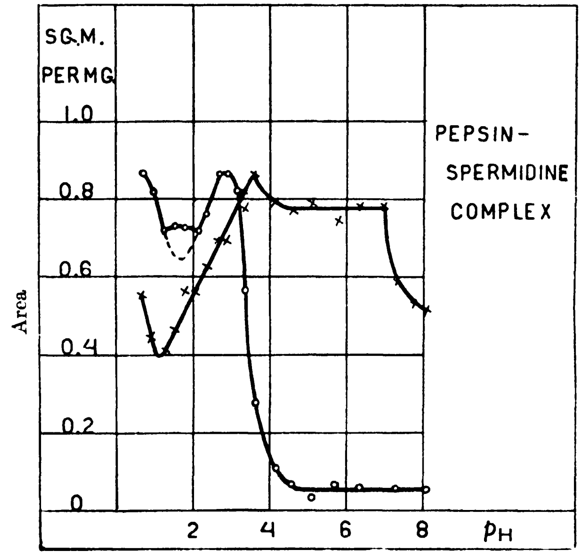


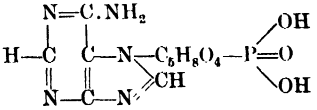
Fig. 3. The curve o—o—o gives the area for the spreading of pepsin (Northrop) on solutions of varying p_H. The dotted line between p_H 1·2 and 2·1 is the curve given by pepsin, to which no acetic acid is added as in this experiment (see experimental part).

The curve ×—×—× gives the spreading of a pepsin-spermidine complex on solutions of varying p_H.

This is to be explained by the transformation of the protein into a multivalent amine.

Here however on the acid side of the isoelectric point a different spreading is also observed with spermidine-pepsin. This may be due to the fact that pepsin is already a complex protein (see below).

We have also studied the influence of nucleic acid on the spreading of ovalbumin,



$$\begin{array}{l} \mathrm{N{=}C.NH_2} \qquad\qquad\quad \mathrm{OH} \\ \mathrm{H{-}C\ \ C{-}N{-}C_5H_8O_4{-}P{=}O} \\ \mathrm{N{-}C{-}N{=}CH} \qquad\qquad \mathrm{OH} \end{array} \qquad \text{(III)}$$

and we were unable to discover any definite change of the spreading at varying p_H as is shown in Fig. 4.

This fact is readily explained by the amphoteric character of nucleic acid itself[1]. At p_H 3·0 on the acid side of the isoelectric point combination of the phosphoric acid groups with the NH_2 groups of the protein is to be expected. But this does not influence the spreading, because the other end of the molecule has a free NH_2 group which simply replaces the original free NH_2 group of the protein. On the alkaline side at p_H 6·0, the same reasoning holds good. The free NH_2 group of the nucleic acid then enters into relation with the free COOH group of the protein, but now the acid character is not modified, the COOH groups being simply replaced by the free acid groups of the phosphoric acid. The same reasoning can explain the absence of different behaviour in spreading experiments with the azoprotein prepared by coupling ovalbumin with the compound

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[1] We give the formula of ¼ part of nucleic acid (III) to show this.

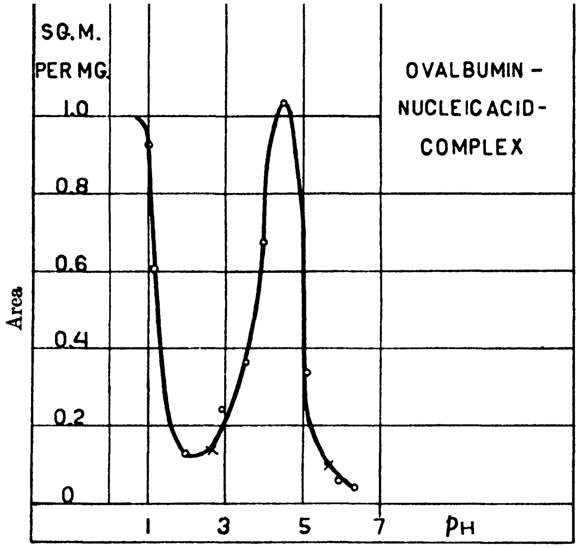
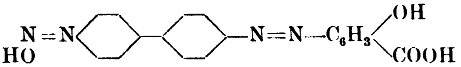


Fig. 4. Nucleic acid in 16*mM* solution is added to ovalbumin. There are only minor differences from the ovalbumin-curve (*cf.* Fig. 1). Points o and × represent different preparations.



$$\underset{\mathrm{HO}}{\mathrm{N{=}N}}{-}\mathrm{C_6H_4{-}C_6H_4{-}N{=}N{-}C_6H_3}\begin{matrix}\mathrm{OH}\\ \mathrm{COOH}\end{matrix}$$

If now we compare the behaviour of this group of complex proteins with that of trypsin, pepsin and insulin, it is possible to state that in the latter proteins only few NH_2 or COOH groups are present.

Pepsin gives the same type of curve as shown by tartrazin-ovalbumin [Gorter and van Ormondt, 1935]; this is in agreement with the small number of free NH_2 groups (5 per molecule of 34,500) found by Northrop [1930] for this protein. Moreover, the curve of acetylpepsin[1] is somewhat modified, the minimum on the acid side of the isoelectric point being less distinct than that of ordinary pepsin (Fig. 5).

The behaviour might be explained by the assumption that pepsin is formed by the combination of a protein with a fairly strong multivalent acid neutralising most of the NH_2 groups of the protein molecule.

The reverse observation can be made with trypsin [Gorter and van Ormondt, 1935] which behaves like a spermidine-protein complex. It is reasonable to suppose that trypsin is a complex protein consisting of a combination of a protein and a multivalent base.

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[1] Kindly supplied by Dr J. H. Northrop.

Insulin [Gorter and van Ormondt, 1933] gives a curve that would indicate that some of the NH_2 groups are not available for reaction with H ions on the acid side of the isoelectric point, because its minimum is not pronounced as in ovalbumin. It has the form of the curve obtained with a tartrazin-ovalbumin complex formed by admixture of less than 5·6*mM* tartrazin solution with a 0·5 % ovalbumin solution.

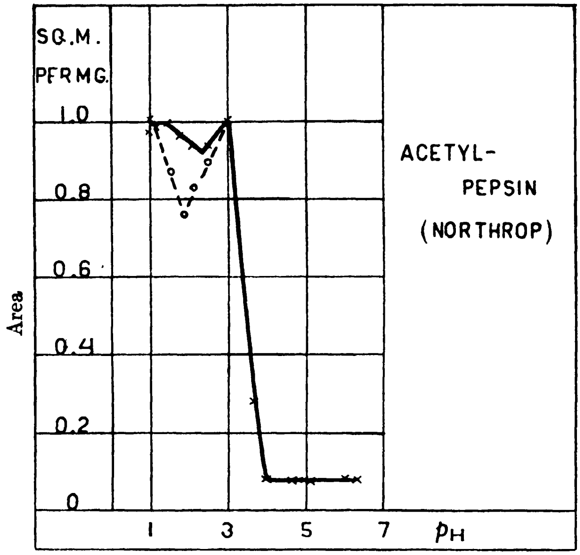

Fig. 5. The spreading of acetylpepsin. The dotted line gives the spreading of pure pepsin and the continuous line that of acetylpepsin solutions of varying p_H.

It is interesting to add to the above-mentioned fact a series of observations showing that not only does ovalbumin combined with tartrazin behave differently from ordinary ovalbumin, but that the same result can be obtained by spreading ordinary ovalbumin at a p_H 3·0 on a tray containing tartrazin.

Here the maximum spreading is obtained within a short time after the ovalbumin has been blown out on the surface of the water in the tray containing tartrazin in a 1/80*mM* solution at p_H 2·7 (Fig. 6), and the same observation holds good at p_H 5·6 when spermidine is added to the water in the tray on which ordinary pepsin is spread, and the spermidine content is *mM* at p_H 5·6 or 7·45 (Fig. 7).

Fig. 8 gives for comparison the amount of spermidine to be added to 0·5 % ovalbumin solution in order to obtain a product that shows maximum spreading at p_H 5·6.

This effect is no longer observable with the tartrazin-ovalbumin or spermidine-pepsin complex owing to the fact that the NH_2 groups or COOH groups can no longer react.

This influence of substances such as tartrazin on the acid side is exactly comparable with the influence of bivalent negative ions (*e.g.* $SO_4^{=}$ or glutathione) whereas spermidine (Figs. 9 and 10) has the same action as bivalent positive ions (*e.g.* Mg, Sr or agmatine). We can obtain maximum spreading of ovalbumin

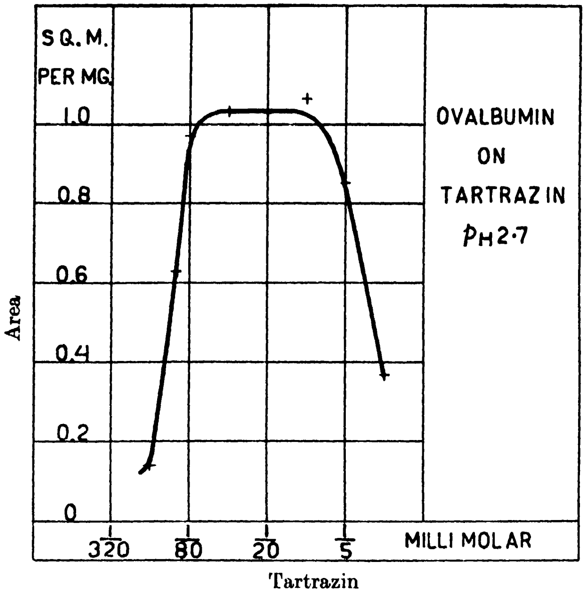

Fig. 6. Showing dependence of maximum spreading of ovalbumin on tartrazin at constant p_H upon the concentration of tartrazin.

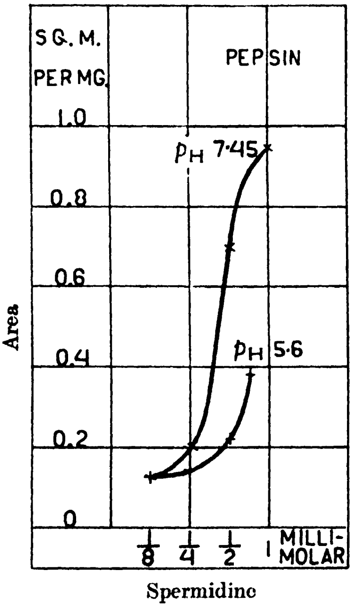

Fig. 7.

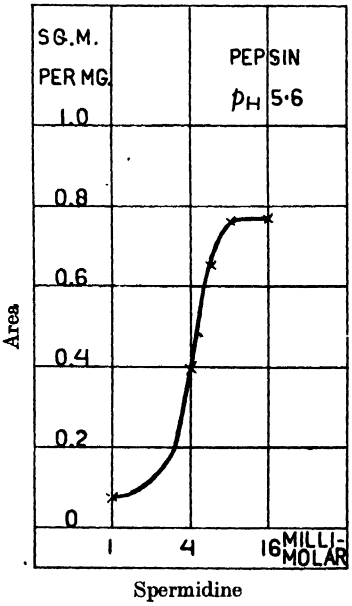

Fig. 8.

Fig. 7. Effect of concentration of spermidine in tray solution upon spreading of pepsin.

Fig. 8. Effect of concentration of spermidine in pepsin solution upon spreading of latter on acetate buffer solution, 0·033 M.

at p_H 3·0 by addition of the negative ions and at p_H 6·0 by adding the positive ions.

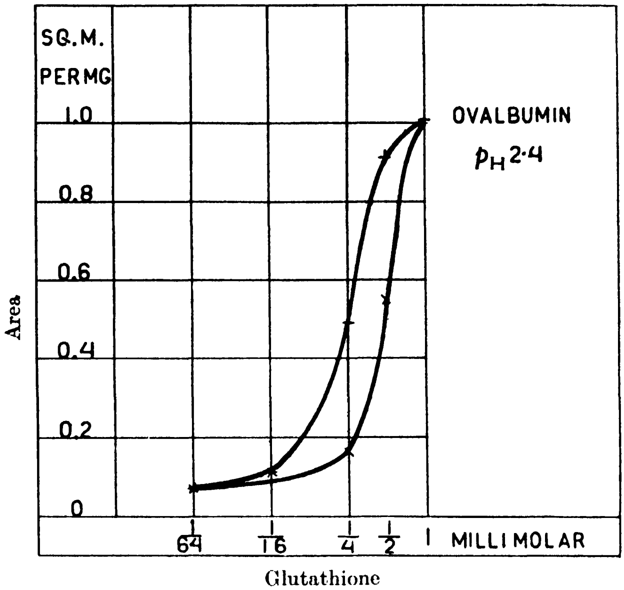

Fig. 9. Spreading of ovalbumin on solutions of glutathione of varying concentration. Left-hand curve after 6 minutes; right-hand curve after 12 minutes.

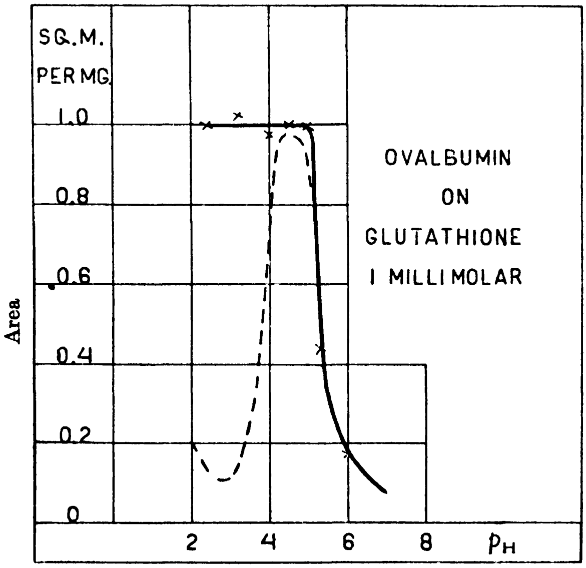

Fig. 10. Continuous curve shows spreading of ovalbumin on glutathione solutions of varying p_H; dotted curve shows the spreading on water at similar p_H.

The above experiments may indicate that at the water surface compounds of the protein and these different substances are formed.

Preparation of ovalbumin.

The ovalbumin used in these experiments was prepared in the following way.

The white of 20 fresh eggs, usually about 600 ml., was beaten and mixed with an equal volume of saturated ammonium sulphate to precipitate ovoglobulin. After standing 24 hours in the ice-box, the ovoglobulin was removed by centrifuging and to the remaining solution was added, slowly with careful stirring, saturated ammonium sulphate until the liquid became distinctly turbid. The slight precipitate was dissolved by slowly adding just sufficient distilled water. To the clear solution was then added, very slowly with careful stirring, 10 % acetic acid until again a slight precipitate was formed. Then some more saturated ammonium sulphate was added to induce rapid crystallisation. After standing 1 or 2 days in the ice-box a large amount of ovalbumin had crystallised. It was centrifuged and dissolved in distilled water. The solution was again treated with ammonium sulphate and acetic acid in the manner described above, and in all six recrystallisations were usually carried out in about a fortnight.

The solution was then dialysed in the ice-box against distilled water, using a cellophane membrane, until no more sulphate could be detected in the outer liquid. The concentration of the pure solution was determined either by ter Meulen's micro-nitrogen method or Bang's gravimetric method.

Preparation of the azoprotein.

0·46 g. of benzidine was dissolved in 100 ml. of water containing 3 ml. of 1 : 1 hydrochloric acid and tetrazotised at 7–8° with an aqueous solution of 0·35 g. of sodium nitrite. The solution was poured into a chilled solution of 3 g. of sodium acetate in 500 ml. of water, and to this was added a solution of 0·36 g. of salicylic acid in 100 ml. of water. The mixture slowly became a chocolate-brown colour, especially after adding 20 ml. of N K_2CO_3. The intensely coloured solution now contained tetrazotised benzidine coupled on one side with salicylic acid, but with the other diazo-group free.

165 ml. of dialysed egg-albumin free from ammonium salts containing about 1 g. of protein were made alkaline with 15 ml. of N K_2CO_3. 130 ml. of the above diazo-solution were then run in followed by 8 ml. of N K_2CO_3.

The dye protein solution was next chilled overnight and acidified first with acetic acid and then with hydrochloric acid until acid to Congo red.

The crude salicylic acid-azo-benzidine-azo-egg-albumin was collected by centrifuging. The centrifugate was washed with acidified water and again centrifuged. This was repeated three times. The precipitate was dissolved in distilled water by adding a few drops of 2·5N NaOH. The p_H of the solution was 11·6; by addition of HCl it was reduced to 8·4.

Preparation of the tartrazin-ovalbumin complex.

Since tartrazin is an acid dye, according to the rule that acid dyes colour best in acid medium [Hammersten *et al.*, 1928], the tartrazin solution and the ovalbumin solution were mixed at p_H 4·0. 4 ml. of egg-albumin solution, containing 60 mg. per ml., were diluted with citrate buffer (McIlvaine) to 44 ml., and to this solution 20 ml. of 1 % tartrazin in 50 % alcohol were added. A yellow precipitate separated and was centrifuged. The precipitate was washed three times with citrate buffer solution (p_H 3·0). The precipitate could be dissolved in a buffer solution of p_H 11·0 (made from 0·1N NaOH and M/10 borax).

The percentage of tartrazin in the solution was determined colorimetrically and the amount of egg-albumin by the micro-Kjeldahl method. The nitrogen content of the solution was determined and from this the nitrogen value of the tartrazin was subtracted.

Ovalbumin with varying amounts of tartrazin, obtained by simply adding different amounts of tartrazin solution to an ovalbumin solution, were made up. The following were prepared: 10 ml. containing 6·2 mg. ovalbumin per ml. and 0·016, 0·008, 0·004, 0·002 and 0·001M tartrazin respectively. The p_H was about 5·7.

Ovalbumin with varying amounts of nucleic acid.

In a similar way solutions containing ovalbumin and adenylic acid or rather sodium nucleate were prepared.

The following were prepared: a solution containing 6·2 mg. ovalbumin per ml., which was 0·016 *M* with respect to adenylic acid; the other solutions contained the same amounts of ovalbumin but were 0·008, 0·004, 0·002 and 0·001 *M* with respect to adenylic acid.

Pepsin with spermidine.

A solution of pepsin [Northrop, 1930] in glycerol containing 30 mg. of crystalline pepsin per ml. was diluted five times, and 1 ml. quantities of this solution were mixed with varying amounts of 0·032 *M* spermidine and the equivalent amounts of acetic acid. Each mixture was made up to 2 ml. The following were the final concentrations of spermidine: 0·001, 0·002, 0·004, 0·008 and 0·016 *M*.

These experiments have been made possible by a grant from the Pilter Langeshuizen Fund.

REFERENCES.

Adam (1930). The physics and chemistry of surfaces. (London.)
Gorter (1926). *Kon. Akad. Wetensch. Proc.* **29**, 1262.
—— (1934). *Kon. Akad. Wetensch. Proc.* **37**, 20.
—— and Grendel (1926). *Trans. Faraday Soc.* **22**, 477.
—— —— (1928). *Biochem. Z.* **201**, 319.
—— —— (1929). *Kon. Akad. Wetensch. Proc.* **32**, 770.
—— and van Ormondt (1933). *Kon. Akad. Wetensch. Proc.* **36**, 922.
—— —— (1935). *J. Gen. Physiol.* In press.
—— —— and Dom (1932). *Kon. Akad. Wetensch. Proc.* **35**, 838.
—— and Seeder (1932). *Kolloid Z.* **61**, 246.
—— Meijer and Philippi (1934). *Kon. Akad. Wetensch. Proc.* **37**, 355.
Hammersten, Hammersten and Teorell (1928). *Acta Med. Skand.* **68**, 219.
Hughes and Rideal (1932). *Proc. Roy. Soc. Lond.* A **137**, 62.
Northrop (1930). *J. Gen. Physiol.* **13**, 739.

VI. THE SPREADING OF MYOSIN.

BY EVERT GORTER AND HANS VAN ORMONDT.

From the Laboratory of the Children's Hospital of the University of Leiden, Holland.

(*Received October 4th, 1934.*)

WHEREAS most proteins so far studied show spreading at their isoelectric points or on strongly acid solutions (we have examined more or less thoroughly ovalbumin, zein, gliadin, insulin, pepsin, trypsin, ovoglobulin, ovomucoid, caseinogen, globin and haemoglobin) some proteins do not spread, when tested by our usual technique. Gelatin is a bad spreader and this is due to its too great solubility. Myosin and probably fibrinogen also do not spread because the particles of the sols have too little solubility.

Spreading of well-purified myosin is very difficult to obtain. At the isoelectric point (p_H 6·1) spreading is never observed, and even on a strongly acid solution p_H 1·0 sometimes no spreading can be detected.

In early experiments when the myosin was not sufficiently freed from myogen (or other contaminants) we obtained some spreading differing according to p_H. When the solutions were almost free from other proteins, we observed small films of protein floating on the surface, visible to the naked eye, and having a bluish tint. After more prolonged purification, this also was no longer observed.

Now it is possible to induce myosin solutions to spread by adding to the solution a trace of a proteolytic enzyme. Before degradation takes place into small particles which have no longer spreading properties (amino-acids never spread and neither do smaller peptides like the tetrapeptide of alanine and the tripeptide of leucine [Gorter *et al.*, 1934]) the myosin is transformed into a spreading substance; this process is highly dependent on the temperature of the solution and on the amount of the trypsin added, and it takes some time before the final effect of the action of the trypsin on the myosin is obtained, when the concentration of trypsin is rather low.

In Fig. 1 the results are given of measurements of the spreading of myosin solutions (6 mg./ml.) to which trypsin in one concentration 1/3250 was added. The temperature was kept constant (38°) and the results after various times were plotted.

It appears that before hydrolysis as indicated by the falling off of the curve occurs, the myosin is transformed into a group of spreading substances. Even during a certain length of time spreading remains maximum, although the enzymic process must be considered as being continuous.

Whereas the curve in Fig. 1 from 2 to 10 minutes appears linear, experiments with smaller amounts of trypsin indicate that this appearance is incorrect (Fig. 2).

Fig. 3 shows the influence of the concentration of trypsin on the spreading of myosin solutions of the same strength, time and temperature being constant (8 minutes and 38° temperature).

The next series of curves (Fig. 4) shows the results of measurements of spreading when different concentrations of trypsin are added to myosin (6 mg./ml.) after various periods of time at the same temperature. If the concentration is high (1/125), no maximum spreading is observed: the hydrolysis has proceeded too far before the first measurement can be made (2 minutes).

In the next series with a concentration of 1/350, hydrolysis begins just after 2 minutes, when the maximum has been reached. At 1/1000 the curve begins

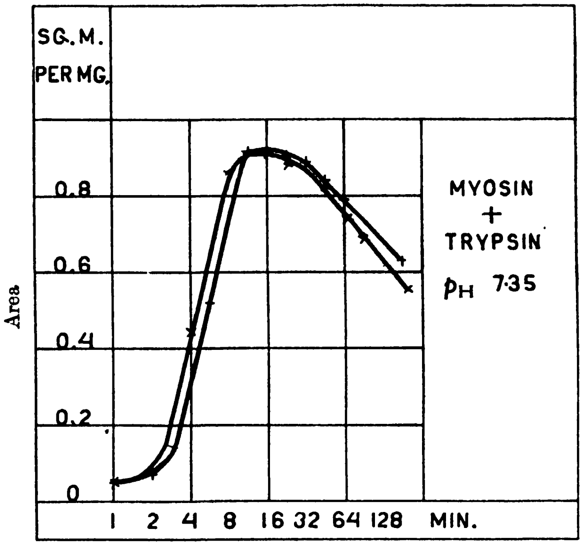

Fig. 1. Influence of time of action of trypsin (final concentration 1/3250) on spreading of myosin (6 mg./ml.). $T = 38°$. Tray solution 0·08 M phosphate.

— x — x — x — Myosin four times reprecipitated.

— + — + — + Myosin three times reprecipitated.

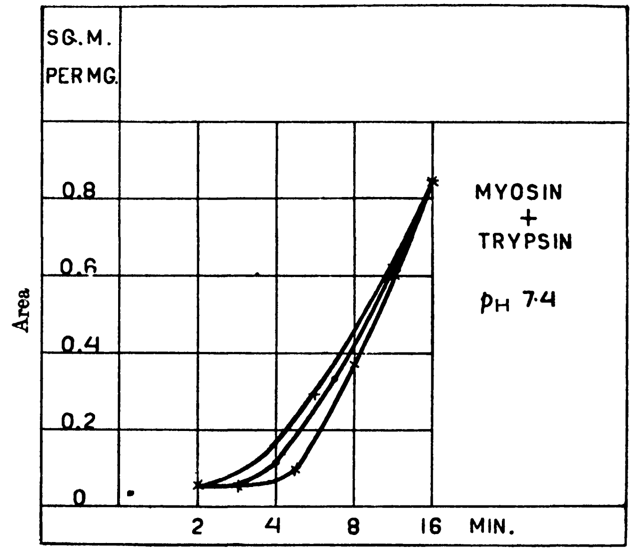

Fig. 2. Same as Fig. 1 save that trypsin concentration was 1/8000. $T = 40°$. Differences between the curve are due to experimental error.

to fall after 4 minutes and at 1/2200 both the beginning of the favourable influence on spreading and of the hydrolysis can be observed; the first process has already proceeded very far towards completion at the time of the first measurement. The typical form of curve showing a gradual rise to a plateau followed by a fall appears at dilutions of 1/8000 and higher.

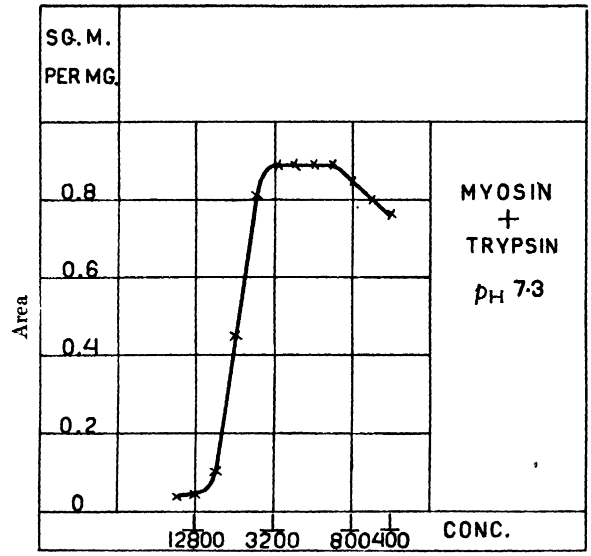

Fig. 3. Effect of varying concentration of trypsin on spreading of myosin. Time of action of trypsin 4 minutes. $T = 38°$.

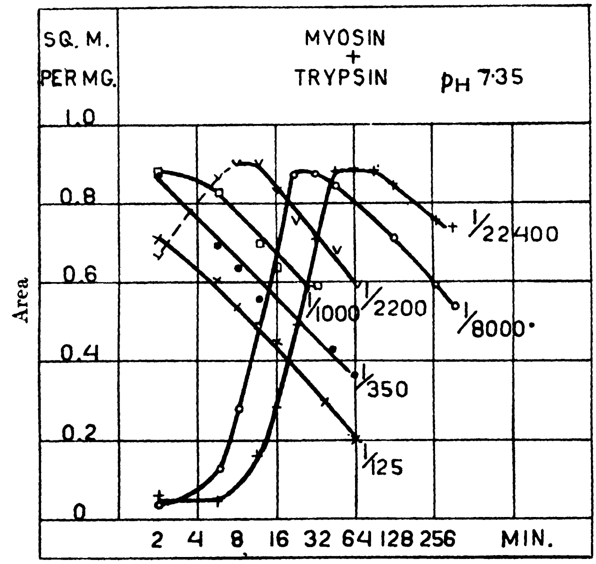

Fig. 4. Curves constructed similarly to those of Fig. 1 but with different concentrations of trypsin. Figures indicate relation between trypsin and myosin; *e.g.* 1/125 means 0·1 mg. trypsin and 12·5 mg. myosin in 2 ml. phosphate buffer.

In order to study the influence of temperature on the first part of the process, we have tried to get the same curve for different temperatures and different concentrations.

Fig. 5 shows that the same type of curve results when spreading is plotted against time. From these observations a linear relationship between temperature and the logarithm of the trypsin concentration can be deduced (Fig. 6) from which a temperature coefficient of 3·6 for 10° can be calculated.

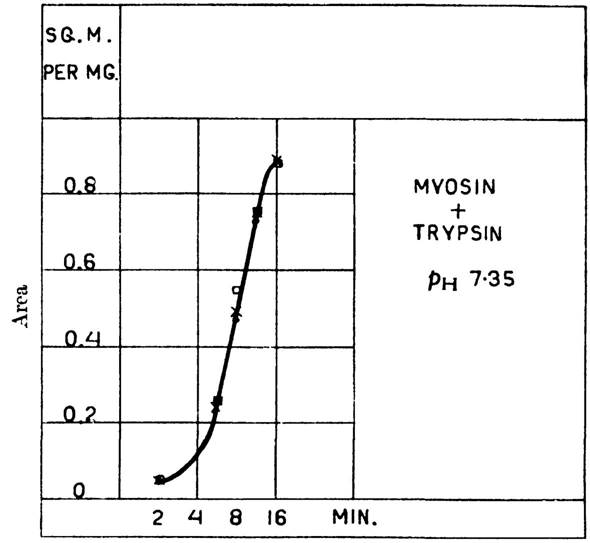

Fig. 5. Showing that the same area for the spreading of myosin after exposure for a given time to the action of trypsin can be obtained by varying both temperature and concentration of trypsin. If temperature is increased, the concentration must be lowered to get the same effect.

+—+—+ $T = 16°$, and trypsin/myosin = 1/350.
×--×—× $T = 24°$. „ „ = 1/1000.
●—●—● $T = 40°$. „ „ = 1/7900.
□—□—□ $T = 48°$. „ „ = 1/22,400.

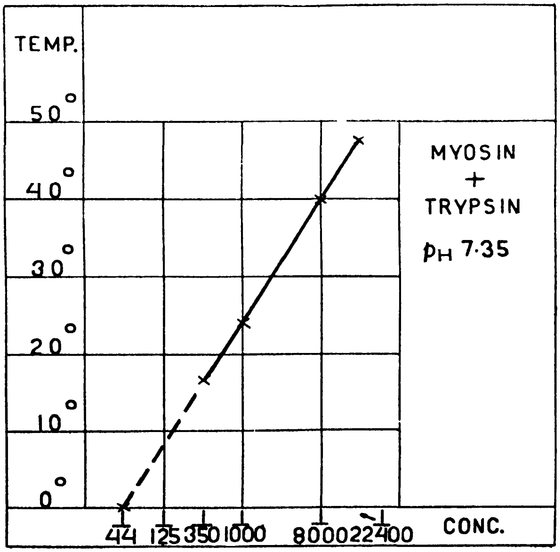

Fig. 6. Showing linear relationship between the logarithm of the concentration of trypsin and the temperature. Temperature coefficient = 3·6. The point at 0° is obtained by extrapolation.

The maximum spreading per mg. myosin under these conditions is 0·9 sq.m. It is somewhat less than the maximum spreading of most other proteins at the isoelectric point.

All the above-mentioned measurements were made on 0·08 M phosphate buffer solution, having p_H 7·3–7·4. It is possible to show that the product obtained by adding trypsin to myosin at the moment when it begins to show maximum spreading is not very much influenced by p_H at about this reaction. We have obtained the same figure when examining "spreading myosin" in the same phosphate solution at p_H 7·1, 7·4, 7·6 and 8·1. This result was obtained by arresting the reaction by cooling the mixture to 0°.

We have also found that a muscle extract, probably containing the proteolytic enzyme from muscle, can in much smaller dilutions produce the same effect on a myosin solution as does trypsin.

The foregoing results seem interesting in so far as they show that an enzyme can, by inducing a protein to increase its surface, prepare it for hydrolysis into smaller peptides and amino-acids.

They have perhaps a still greater importance in other respects, because they show that there exist proteins which do not spread under the conditions that permit a spreading of most other proteins (at p_H 1·0, at the isoelectric point or after addition of bivalent negative ions on the acid side or of positive ions on the alkaline side of the isoelectric point), but that partial hydrolysis into simpler compounds is necessary to produce this spreading.

It is worth while to emphasise that this behaviour is observed with a protein, which shows birefringence of flow under polarised light, which is a proof of the existence of long-shaped particles in a myosin solution.

Preparation of myosin.

The myosin used in these experiments was prepared in a way similar to that indicated by Edsall [1930].

A rabbit was narcotised by means of a 20 % solution of urethane, injected intraperitoneally. The animal was bled to death by opening the carotid artery. The muscles of the legs and back were taken, weighed and quickly put through a mincer. The minced muscle was brought into about three times its weight of a concentrated salt solution containing 1·2 M KCl, 0·05 M Na_2HPO_4 and 0·01 M KH_2PO_4. The mixture was kept at 0° and stirred vigorously for about 2 hours, during which time it became very viscous. It was strained through a linen cloth. The undissolved part was again mixed with salt solution (about a third of the quantity used the first time) and extracted at 0° during 2 hours. This was repeated until the solution no longer showed double refraction of flow and gave only a slight precipitate with salicylsulphonic acid. Usually three or four extractions sufficed.

The different solutions of muscle protein were combined and added slowly with careful stirring to twenty times their volume of ice-cold distilled water. The precipitate was centrifuged, redissolved in the smallest possible amount of the above-mentioned salt solution and the myosin reprecipitated by pouring into 20 vols. of ice-cold water. In this way the myosin was purified by three or four reprecipitations. The process of purification could be controlled by the spreading experiments: after some precipitations the product no longer spread, either immediately or after standing for an indefinite length of time.

The concentration of the solutions was estimated by means of micro-Kjeldahl determinations.

These experiments have been made possible by a grant from the Rockefeller Foundation.

REFERENCES.

Gorter, Meijer and Philippi (1934). *Kon. Akad. Wetensch. Proc.* **37**, 355.
Edsall (1930). *J. Biol. Chem.* **89**, 289.

VII. BRAIN RESPIRATION, A CHAIN OF REACTIONS, AS REVEALED BY EXPERIMENTS UPON THE CATATORULIN EFFECT.

BY RUDOLPH ALBERT PETERS, HÅKAN RYDIN[1]
AND ROBERT HENRY STEWART THOMPSON[2].

From the Department of Biochemistry, Oxford.

(*Received November 2nd, 1934.*)

PREVIOUS work has shown that vitamin B_1 acts *in vitro* in respiring enzyme systems from the avitaminous pigeon's brain to induce an increased oxygen uptake [Gavrilescu *et al.*, 1932] in lactate and pyruvate solutions under defined conditions. Pyruvate was formed in lactate solutions in absence of vitamin and disappeared in its presence, suggesting that it was a normal metabolite [Peters and Sinclair, 1933; Peters and Thompson, 1934, 1]. The average value for the quotient, extra O_2 uptake in presence of vitamin/pyruvic acid disappearing, represented 2 mols. of O_2 to 1 mol. of pyruvic acid used, but careful study of this ratio showed variations sufficiently wide to lead to the tentative conclusion that pyruvate disappearance was actually an indirect effect of the added vitamin. In this paper, more evidence is produced in the same sense. The action of the vitamin depends to a marked extent upon the method of preparing the tissue; it is apparently concerned with formation of some unidentified substrate which then interacts to produce increased O_2 uptake. Hence we must visualise the extra oxygen uptake observed as being the result of a chain of chemical events in the tissue.

We have continued to use the avitaminous pigeon's brain for this work; the behaviour of mammalian brain is of course more interesting from many points of view, but use of the pigeon's brain is advantageous in view of the exact knowledge of conditions of avitaminosis and above all of the ease of removal of the brain from the soft skull. It is doubtful whether the facts given below could have been elucidated without the use of the avitaminous brain, which illustrates the advantage of material of this type in illuminating obscure phases of metabolism.

Further details of technique.

Birds used have been in acute opisthotonus symptoms; they have usually been under vitamin B_1 test for 2–8 days, so that the symptoms have appeared for a second time. Depletion is thus more complete. Unless otherwise stated the tissue has been prepared upon a warm plate at 38°; division is done with a small, blunt bone spatula (to avoid cutting and metals). Duplicates are filled alternately; the tissue in the bottles, after re-weighing, is divided with a glass crusher. It is best to allow to stand 3 mins. before making the additions of the vitamin *etc.* Observations have been made in the differential apparatus. Tissue has been put into previously tared bottles. For the rapid simultaneous handling of a large number of duplicate samples from brain tissue, this method has proved more suitable than attempts to weigh exact amounts of tissue upon a balance. All bottles have been filled with oxygen, and duplicates evacuated in the order 1*a*, 2*a*, 3*a*, 4*a*, the first lot, then 1*b*, 2*b*, 3*b*, 4*b*, the second lot, *a* and *b* being duplicates, *etc.* Other details of the work have not been changed.

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[1] Rockefeller Fellow.

[2] Senior Demy, Magdalen College.

Unless otherwise stated, sodium pyrophosphate (at p_H 7·3) has been used throughout, made up so that an addition of 0·1–0·2 ml. of Na pyrophosphate produced a final concentration of 0·01 *M*. Small variations in this concentration do not seem to matter. As has been shown previously [Peters and Sinclair, 1933], this compound enhances the catatorulin effect.

Effect of temperature of preparation upon the rate of respiration[1].

The difficulty of obtaining satisfactory duplicate values for respiring brain tissue "brei" has repeatedly forced itself upon our attention. It seemed also that some general factor of unknown nature influenced the level of respiration, apart from the action of the vitamin. In particular, there was a tendency for lower values in winter. This led us to study the effect of temperature of mincing upon the subsequent rate of respiration, with remarkable results. Some of these have been recorded elsewhere [Peters and Thompson, 1934, 2].

In these experiments, equal halves of the brain of a guillotined pigeon were removed with minimum injury; without delay the halves were minced respectively for 2 mins. upon a plate cooled in ice and upon a glass hot water funnel kept warm at 38° ± 2°. In most cases right and left cerebral hemispheres were compared. The results are given in Table I, and a particularly striking case which is diagrammatic of the effect is shown in Fig. 1.

For the normal brains in 13 out of 15 experiments (see a selection of these in Table I and Fig. 1), there was found to be a large difference between the respirations of the "warm" and "cooled" tissue in lactate solutions. This also occurred in pyruvate solutions with avitaminous brains. On the other hand the tissue in Ringer phosphate solution only (residual respiration) showed little difference. Respiration in succinate again was little affected. Among these three substrates (the only ones tried), the influence was confined to lactate and pyruvate. As in the catatorulin effect, succinate respiration is hardly influenced [*cf.* Gavrilescu *et al.*, 1932]. It might be expected that the tissue prepared "warm" would show damage to its respiration, owing to inactivation by enzymes of some essential constituent. The result is quite different. The greater respiration occurs in the tissue prepared "warm." Since there is little change in residual respiration, it is not merely an increase in tissue substrates. In older parlance, it would have been said that more lactate and pyruvate oxidases are present in the "warmed" than in the "cooled" specimen; but an increased O_2 uptake does not seem to be necessarily associated with increased disappearance of lactate [Meiklejohn, 1933]; hence it is more probable that some essential element of the carbohydrate respiration is retained in the "warmed" brain. It is to be noted that the effect is present with added pyrophosphate.

The difference in respiratory behaviour is accompanied by a visible difference in the state of the tissue; in the tissue kept "warm" the trace of blood present at the end of the period of mincing (2 mins.) is strongly reduced, whereas in that minced "cool" it is a bright red colour; it has also been our impression that the tissue kept "warm" has a slight yellowish tinge. The obvious suggestion that the effect was due to the state of the glutathione has been tested. Exp. 658 shows that the extra respiration is not restored to the "cooled" tissue by addition of glutathione.

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[1] By use of the term respiration, we wish to imply that the oxygen uptake observed is a complex.

Table I. *Comparison of rates of respiration for tissue prepared under "warm" and "cool" conditions.*

Expressed as μl. O_2/g./hr. (wet weight). 2000 = Q_{O_2} 10.

Exp.	Substrate	Prepared	0–½	½–1	1–1½	1½–2
506	Nil	Cool	950	700	530	—
	,,	Warm	1460	930	800	—
	Lactate	Cool	2030	1510	1365	—
	,,	Warm	3060	2330	2280	—
509	Nil	Cool	940	705	585	425
	,,	Warm	1165	830	595	575
	Lactate	Cool	1950	1395	1300	990
	,,	Warm	2370	1880	1500	1380
511	Nil	Cool	655	600	394	364
	,,	Warm	830	615	525	424
	Lactate	Cool	1750	1455	1380	1260 (pp)
	,,	Warm	2255	1790	1708	1663
669	Lactate	Cool	2660	1870	1390	—
	,,	Warm	2870	2410	1790	—
	Succinate	Cool	4610	3900	2900	—
	,,	Warm	4200	3270	2470	—
670	Lactate	Cool	2270	1690	1300	1100
	,,	Warm	3100	2700	2190	1840
	Succinate	Cool	4610	3410	2420	1670
	,,	Warm	4030	3270	2440	1930

Note. The above experiments are illustrative only. 511 was made with addition of pyrophosphate (pp). In 669 and 670 the periods are 0–¼, ¼–¾ hours, *etc.* In 669 and 670 concentration of succinate = 0·07 *M* (25 mg. in 3·0 ml.). Nil = Ringer phosphate only.

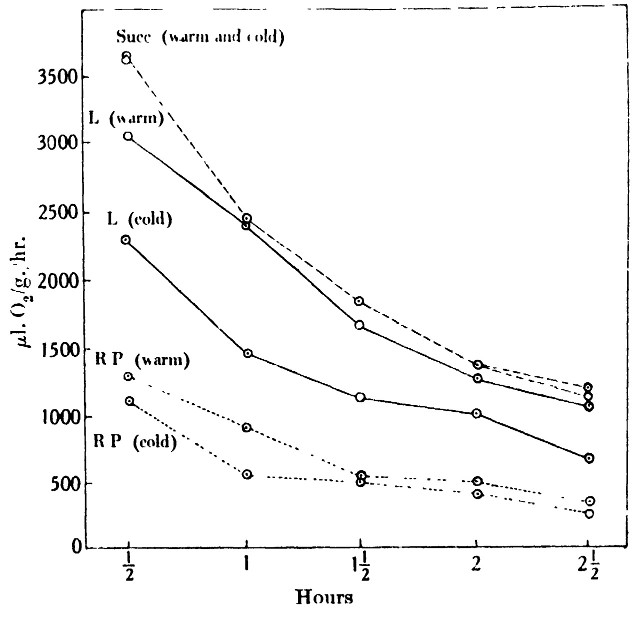

Fig. 1. Exp. 596. Basal medium Ringer phosphate, RP. Succ. = RP + Na succinate, 0·07 *M*. L = RP + Na lactate, 0·03 *M*. Pigeon's brain tissue minced "warm" and "cold". The difference in respiration rates is approximately the same for RP and Succ., but markedly different at first for L.

Exp. 658. The right and left lobes of a pigeon's brain (normal) were removed within 30 secs. of death and minced for 2 mins. upon warmed and cooled plates. Respiration μl./g./hr. Glutathione 0·009 *M*. Ringer-phosphate-pyrophosphate-lactate.

Period (hrs.) ...	¼–¾	¾–1¼	1¼–1¾
Cooled	1760	1470	1370
„ +G	1860	1560	1400
Warmed	2330	2000	1770
„ +G	2460	2160	1855

The slight effect here can be entirely explained upon the grounds of the O_2 uptake of the glutathione itself. Another possibility was tested, *viz.* that part of the effect might be due to drying of the "warmed" tissue and settling of moisture upon the "cooled," though this is really excluded by the succinate experiments; the water contents were found to be not significantly different. It is known [Kinnersley and Peters, 1929] that lactic acid production takes place with great rapidity in these brains, being complete in about 90 secs. At first it was thought that the effect was more marked in brains removed within 30 secs., but this could not be proved.

From the general relation of the catatorulin effect to pyruvate and lactate, it would be inferred that the vitamin would show greatest action upon tissue minced "warm." The experiments, of which one example of each is given in Table II, show that this is so. In one case even there was no effect of vitamin in the "cooled" tissue.

Table II. *Avitaminous brains. Temperature effects.*

μl./g./hr.

Exp.	Substrate	Prepared	Vitamin	¼–¾	¾–1¼	1¼–2¼	2¼–2¾
651	Lactate	Cool	Control	1350	1080	920	980
			V	1295	1145	1050	970
			Diff.	—	65	130	—
		Warm	Control	1905	1620	1320	1095
			V	2140	1955	1730	1540
			Diff.	235	335	410	445
518	Pyruvate	Cool	Control	670	490	370	340
			V	908	686	540	510
			Diff.	238	196	170	170
		Warm	Control	935	670	470	460
			V	1080	960	800	790
			Diff.	145	290	330	330

It is especially difficult to obtain good duplicate values with the "cooled" tissue, probably because of the uneven temperatures induced, even with the best efforts to crush immediately. It is clear that scrupulous attention to minute detail is necessary to get reliable control of these enzyme systems from brain. The temperature effect certainly explains some of our puzzling difficulties in the past.

In previous work, as an experimental hypothesis, we have postulated a substrate *X* with which the vitamin interacts. We have now reached the position that the vitamin effect depends in addition upon some unknown factor present in the "warmed" tissue; it therefore depends upon the method of preparation. If the tissue minced "warm" is transferred to the cooled plate, a change in colour takes place and the tissue becomes bright red. This is followed by a fall in the lactate respiration. Hence cooling and change in the level of oxidation together lower the respiration. On the other hand, if the "cooled" tissue is transferred after mincing to a warmed plate, change of colour indicating reduction occurs but there is no (or very little) return of the respiration rate in lactate or of increased vitamin effect, as we have found in four experiments of which Exp. 664, Table III, is one; compare with Exp. 651, Table II.

Table III.

Exp. 664. Avitaminous brain (cerebellum). Medium lactate-pyrophosphate (M) ± vitamin. Brain tissue removed within 1 min. and thoroughly minced upon cooled plate for 2 mins., colour bright red. Half transferred to warmed plate (38°), and reminced, colour dark purple. Respiration μl./g./hr.

Condition		¼–¾	¾–1¼	1¼–3¾
Cooled	M	1050	675	645
	M + V	1275	960	915
Cooled, then warmed	M	1190	865	795
	M + V	1355	1025	1000

In effect then a mild degree of cold destroys an essential factor, irreversibly. This may be by physical separation, which is rather the opinion of the Warburg school as to the difference in glycolysis *etc.* between sections and tissue pulp. A more attractive view is that there is irreversible oxidation of some essential co-enzyme, when the action of the dehydrogenases is depressed by the cool conditions.

Evidently determinations of Q_{O_2} in tissue "brei" must be influenced by these findings. In previous papers from this laboratory (except the last) the mincing was carried out at room temperature, and the values must be a mixture of the "cool" and "warm" values. The conclusions are not influenced, as they are based upon comparative work.

The catatorulin effect as a chain of reactions.

Until now the simple hypothesis that the vitamin acted upon a substance *X* with the direct result of increased oxygen uptake was tenable. A new experiment of different type disproves this simple view.

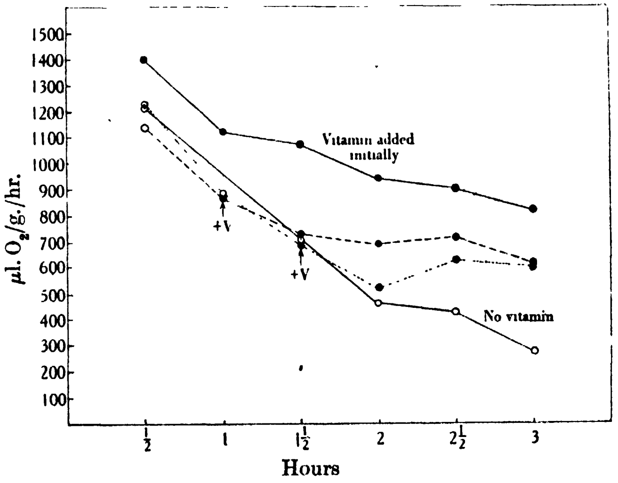

Fig. 2. Exp. 614. o No vitamin. • Vitamin present. Arrow indicates time of addition of vitamin. Avitaminous brain as usual.

Table IV.

Exp. 614. Vitamin added after commencing respiration.

Hours ...	0–½	½–1	1–1½	1½–2	2–2½	2½–3
A. Control	1215	—	710	460	430	270
B. Vitamin	1400	1120	1070	940	900	820
C. Vit. at 1 hr.	1140	870*	722	690	715	610
D. Vit. at 1½ hrs.	1225	885	690*	515	640	600

Note. All contained initially Ringer-phosphate-pyrophosphate and lactate. Vitamin was added to B before commencing the experiment, and to C and D at the times indicated by the asterisks. Dixon-Keilin apparatus used.

It has been realised for a long time in this laboratory that vitamin added at some interval after the start of respiration checks the fall in respiration but produces no rise. In unpublished work, Meiklejohn, Passmore and Peters found a tendency for the rate to be stabilised by added vitamin.

A more recent experiment shows this clearly.

In these tissue "brei" from the avitaminous bird, lactate, lactate + pyrophosphate, lactate + pyrophosphate + vitamin have increasing effects in mitigating the fall in respiration, as well as in increasing the initial rate when added initially.

Of the possible interpretations of these facts we were most attracted by the view that the "stabilised" respiration was due to the formation of some substance during the first 1–2 hours of respiration, which was subsequently oxidised. This was tested in the following way; avitaminous brain tissue was allowed to respire for periods up to $2\frac{1}{4}$ hours in Ringer-phosphate-pyrophosphate ± vitamin B_1, after which lactate was added. Exps. 644 (lactate) and 645 (pyruvate) given in Table V, and 645 (lactate) in Fig. 3 illustrate the results obtained. Delayed addition of lactate (or pyruvate) shows a comparatively small increase in respiration with tissue incubated without vitamin, but a large increase with tissue incubated in the presence of vitamin.

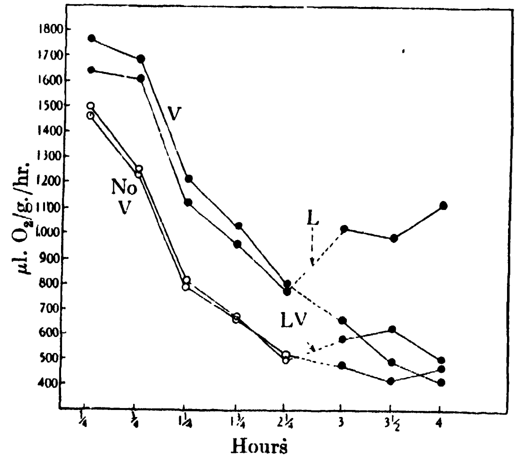

Fig. 3. *Delayed substrate experiment.* 0–2¼ hours' respiration in Ringer-phosphate with and without vitamin. At 2¼ hours, additions of L and LV made to two bottles, and of V to one of remaining bottles. Rise is large only when L is added to tissue previously treated with V. L = lactate. V = vitamin 2γ. ● Vitamin present. ○ No vitamin present. Tissue weights, 340–360 mg. Pyruvate and lactate both used, for pyruvate see Exp. 645, Table V.

Table V. *Delayed type experiments.*

Exps. 644 and 645. Avitaminous brain (cerebellum), prepared warm. Medium (M) Ringer-phosphate + sodium pyrophosphate (p_H 7·3). Vitamin 2γ in Ringer-phosphate-pyrophosphate. After shaking for 2¼ hours bottles were removed, additions made, bottles re-filled with oxygen, and replaced in bath. Time 5–10 mins. Readings commenced after a further 12 minutes' shaking. Solutions adjusted to make final volume 3·0 ml. Lactate added in 0·1 ml. to make 0·033 *M*.

Exp. 644. Respiration μl./g./hr.

	Tissue weight mg.	Hours					Additions	Hours			
		0–¼	¼–¾	¾–1¼	1¼–1¾	1¾–2¼		2¾–3	3–3½	3½–4	4–4½
M only	156	1240	910	755	535	490	LV	545	515	515	525
	184	1310	1045	760	585	460	V	365	375	280	240
M + V	147	1465	1225	1015	800	650	L	855	915	810	800
	194	1515	1270	1090	885	745	Nil	555	425	430	365
Exp. 645. Pyruvate (for lactate see Fig. 3).											
M only	325	1420	1105	745	610	465	PV	440	460	495	—
	267	1300	1050	680	535	420	V	420	360	395	—
M + V	346	1760	1620	1225	1025	821	P	940	955	990	—
	308	1710	1590	1660	980	820	Nil	560	510	430	—

Differences in respiration after the additions.

644	LV–V	180	140	234	285				
	L–Nil	300	491	380	437				
	Diff.	120	351	146	152				
645	LV–V	107	208	42	—	PV–V	20	100	100
	L–Nil	365	492	700	—	P–Nil	380	445	460
	Diff.	258	284	660	—	Diff.	360	345	360

Note. To get satisfactory results, this experiment requires larger amounts of tissue than usual. It is better to use two brains, or, if unavailable, one cerebrum distributed in four bottles. In the latter and more usual case, duplicate estimations are not possible. The experiment was sometimes done in the Dixon-Keilin apparatus, but it is quite effective in the usual type of bottle. The differences in respiration are very large; the larger amounts of tissue complicate the issue with vitamin at the beginning owing to the larger amount of residual lactate present, but in most cases the rise is beyond experimental error.

This experiment has been repeated many times (25) with essentially the same results, though the rise is variable. Pyruvate can be substituted for lactate, and pyrophosphate when present in the incubation period enhances the effect.

In several of the experiments, the rise upon addition of lactate is proportional to the value for the respiration immediately before making the addition. In others the rise for the vitamin-treated samples is sufficiently large to exclude this interpretation. Hence something is present in the vitamin-treated sample which develops an oxygen uptake only when treated with substrate.

In the "delayed substrate" experiments the respiration increases upon adding lactate. Hence, the vitamin cannot be merely preventing the disintegration of the respiratory system [*cf.* Peters and Sinclair, 1933]. But there must be an accompanying inactivation of some essential co-enzyme, since the respiration after 2 hours never recovers to more than 1200–1400 μl., a value considerably less than the initial respiratory value.

A new hypothesis which embraces these further facts is as follows:

Stage 1 Stage 2
$X \longrightarrow Y$ + Pyruvate or lactate and O_2 = Products of oxidation and oxidative synthesis
$\uparrow$
Vit. B_1
Pyrophosphate

Stage 1 requires little if any extra O_2, and is therefore likely to be a "dehydrogenase" stage. Stage 2 needs the presence of the co-enzyme injured by oxidation. *X* must be regarded as a precursor of *Y*.

It is quite possible that *Y* is merely an active state of *X*, stabilised by vitamin B_1-pyrophosphate, or indeed that the vitamin merely prevents the relapse of *Y* into an inactive state.

If this hypothesis is correct, we should find marked differences in the responses to lactate of "warmed" and "cooled" tissue after the incubation period, without much effect previous to lactate addition. Exps. 660 and 661 in Table VI show that this is so (two similar experiments are not quoted). It is remarkable how much the delayed vitamin effect is increased in the "warmed" tissue. The initial vitamin effect in the "warmed" is accounted for by the presence of residual lactate.

Table VI. *Delayed addition of substrate; "warm" and "cooled" tissue.*

Exp.	Period (hrs.)	...	0–¼	¼–¾	¾–1¼	1¼–2¼	2¼–2¾	↓	3–3¼	3¼–3¾	3¾–4¼	4¼–4¾
660	Cooled	Rpp	1175	835	700	500	380		760	560	450	410
		„ +V	1280	915	730	540	385		785	750	690	630
							Diff.		25	190	240	220
	Warm	Rpp	1755	1260	970	730	495	↓	950	750	650	550
		„ +V	2040	1430	1110	760	540		1245	1255	1185	1145
							Diff.		295	505	535	595
661	Cooled	Rpp	1520	1024	845	695	525	↓	805	600	515	445
		„ +V	1325	1040	920	850	560		1100	885	815	780
							Diff.		295	285	300	335
	Warm	Rpp	1790	1605	1085	840	610	↓	910	700	630	530
		„ +V	2080	1790	1365	1015	690		1240	1240	1225	1155
							Diff.		330	540	595	625

Note. Rpp = Ringer-phosphate-pyrophosphate medium. V = 2γ vitamin B_1. Lactate added at the arrow (0·1 ml. to make 0·033 *M*). In each of these experiments separate halves of the cerebrum were removed as quickly as possible with minimum injury and minced for 2 mins. respectively on "warmed" and "cooled" plates; colour of "warmed" tissue was purple and of "cooled" bright red. Addition of vitamin was made after weighing and subdivision of the tissue.

The nature of X[1] *and* Y. Upon the present hypothesis, a substance should behave as *Y* if it interacts immediately upon addition of lactate to produce a rise in respiration; it cannot be α-glycerophosphate, because this was shown to be independently additive in oxygen uptake experiments by Peters and Sinclair. The "delayed" experiment does not decide whether vitamin B_1 is essential for stage 2.

A substance will substitute for *X* if it causes a delayed increase in respiration when lactate + V are present, or increases the rise upon lactate + vitamin addition in the "delayed" experiment.

Several obvious brain constituents have been tested in the attempt to identify the unknowns. So far no effect of the required type has been seen with creatine, creatinine, inositol, galactose, xanthine or crude hexosediphosphate (candiolin). Several experiments with extracts of brain have given suggestive, but not decisive, results. Some *X* appeared to be present in a neutralised trichloroacetic extract of hen's or pigeon's brain.

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[1] A possible alternative hypothesis. We have given what appears to be the simplest interpretation of our facts. It is just possible that the facts can be explained without recourse to the "indirect" oxidation view. If the vitamin in oxidising the residual lactate maintains the stability of the essential factor, we can then explain the delayed type of experiment as being merely the result of better protection of the essential factor from irreversible destruction. In this case, there would be no need to postulate the substance *X*.

DISCUSSION.

These experiments upon brain tissue depleted of vitamin B_1 demonstrate that oxidations (in the sense of oxygen uptake) with added lactate or pyruvate are secondary to a preparative stage, which is presumably one of H transfer. They seem fundamental, because the oxidation system of lactate is involved. As has been previously suspected, tissue respiration involves an organised chain of reactions, even in a "brei" of this nature.

We have been led by another path to the technique of Quastel and Wheatley [1932] upon which they base the conclusion that narcotics influence the oxidation of lactate rather than succinate. This conclusion is strikingly coincident with the independent finding of Gavrilescu *et al.* [1932], that the nervous symptoms in avitaminosis are related specifically to a lactate rather than a succinate lesion. The finding of Gavrilescu *et al.* can however be demonstrated upon freshly removed tissue. The technique of Quastel and Wheatley [1932] involves a preliminary autoxidation of the brain tissue for periods up to 3 hours, after which the substrate is added. It seems clear now that the subsequent reaction of the tissue to substrate will depend upon some action of vitamin B_1 (and possibly other factors) in the preparative stage.

Since the narcotic (or poison) is added during the preparative stage, the narcotic may well be influencing this preliminary stage, and no conclusions can really be drawn about the effect upon the oxidative stage. This is borne out by experiments upon the effect of fluoride and iodoacetate in the delayed substrate experiment in an accompanying paper [Peters *et al.*, 1935]. Hence the interpretation of Quastel and Wheatley might require modification to the extent that narcotics influence a constituent in the "coupled" oxidation of lactate[1]. That the "coupled" oxidation of lactate might be so influenced at a dehydrogenase stage by narcotics is satisfactory because it brings these effects into line with a generalisation of Keilin [1929] that narcotics in general interfere with the reduction of cytochrome by dehydrogenases.

Much has been made of the very real difference between the behaviours of tissue sections and "brei" in many types of experiment. The writers suggest that in sections the co-enzyme present especially in "warm" minced brain is better protected from irreversible oxidation by association with an active dehydrogenase system, which is more complete.

In regard to the specific application to vitamin B_1 problems, the possible interpretations discussed by Meiklejohn of his failure to correlate extra oxygen uptake of the vitamin with increased disappearance of lactate were three. Of these the most acceptable seems to be that vitamin catalyses the coupled oxidation in presence of lactate of some other substance. It is proved here that pyruvate can replace lactate; presumably the removal of "avitaminous" pyruvate is secondary to the action of vitamin B_1. Unfortunately, we cannot yet settle whether pyruvate or lactate is immediately concerned in the interaction. We must further point out that the proof that vitamin B_1 directly catalyses metabolism of some carbohydrate constituent is still lacking in absence of knowledge as to the exact nature of Y, though there is abundance of evidence as to the indirect connection with carbohydrate metabolites.

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[1] It is possible that ignorance of these facts is the source of controversy between Bülow and Holmes and Quastel and colleagues; but see a recent paper by Quastel and Wheatley published while this paper was in course of preparation.

SUMMARY AND CONCLUSIONS.

1. As compared with tissue prepared "cold," pigeon's brain tissue prepared "warm" shows marked differences in oxygen uptake. A higher respiration is found in lactate and pyruvate solutions with the "warmed" tissue, whereas only slight differences occur in Ringer phosphate and succinate solutions.

2. The catatorulin effect of vitamin B_1 is most marked with tissue prepared "warm."

3. Vitamin B_1 interacts with some unknown substrate to produce a substance which increases the oxygen uptake in the presence of lactate or pyruvate. The catatorulin effect therefore depends upon a chain of tissue reactions.

4. Sodium pyrophosphate influences the interaction with the unknown substrate.

5. The following hypothesis for the oxidation system is suggested:

Stage 1 Stage 2
$X \longrightarrow Y$ + Pyruvate or lactate and oxygen = Products of oxidation and oxidative synthesis
↑
Vitamin B_1. Pyrophosphate
(depressed by fluoride
or iodoacetate)

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REFERENCES.

Gavrilescu, Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **110**, 431.
Keilin (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.
Kinnersley and Peters (1929). *Biochem. J.* **23**, 1126.
Meiklejohn (1933). *Biochem. J.* **27**, 1310.
Peters and Sinclair (1933). *Biochem. J.* **27**, 1910.
—— and Thompson (1934, 1). *Biochem. J.* **28**, 916.
—— —— (1934, 2). *J. Soc. Chem. Ind.* **53**, 453.
—— Rydin and Thompson (1935). *Biochem. J.* **29**, 63.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 725.

VIII. THE RELATION OF PYRUVIC ACID IN BRAIN TO CERTAIN TISSUE POISONS.

By RUDOLPH ALBERT PETERS, HÅKAN RYDIN[1]
AND ROBERT HENRY STEWART THOMPSON[2].

From the Department of Biochemistry, Oxford.

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Some indication was obtained by Peters and Thompson [1934] that fluoride diminished the substantial amount of pyruvate formed by avitaminous brain, as well as the smaller amounts formed in normal brain respiration. On the other hand sodium iodoacetate led to increase of pyruvate even in normal brain: consideration of this for avitaminous brain was left until more data became available. This study was undertaken in the hope of deciding the point of action of vitamin B_1 and also of testing further the applicability of the Embden-Meyerhof scheme to brain systems. According to Meyerhof and Kiessling [1933], iodoacetate and fluoride inhibit different stages in the fermentation cycle; fluoride inhibits the appearance of pyruvic acid from phosphoglycerate and iodoacetate the disappearance of the former; the action of each gives rise to inhibition of lactic acid formation.

Oxygen uptake.

Table I (*a*) summarises the experiments which we have made upon respiration in the presence of the two poisons. The results are given as averages. It is to be understood that in each series of experiments the control and poison have been tested in duplicate upon the same brain. The results for fluoride confirm and extend those of Peters and Sinclair [1933]; the concentration used (0·024 M) is taken from their work. With iodoacetic acid the most suitable concentration was found to be 0·00054 M (0·1 mg. per ml.); this is the amount found to inhibit the action of glyoxalase in tissue sections [Dickens, 1933].

In orientating experiments upon normal brain, 0·0009 M seemed maximum, 0·00018 M gave less effect. With avitaminous brain in four experiments, 0·00018–0·00027 M gave approximately the same effect, 0·000095 M gave sometimes less.

The following can be concluded from Table 1 (*a*). Both poisons reduce the oxygen uptake in lactate solutions; the percentage of respiration so inhibited increases during the period studied, but the absolute amounts inhibited in μl./g./hr. remain surprisingly constant, if we exclude the first period. For avitaminous brain the inhibition amounts to 500 μl./g./hr. in every case except that of fluoride and pyruvate, where the average was 150. This curious difference with pyruvate is probably related to the observation of Meiklejohn *et al.* [1932] that there is a tendency for respiration of avitaminous brain to be lower in pyruvate solutions. The effect is not constant, because in two further experiments carried out recently by the latest technique, in which the actions of fluoride and iodoacetic acid with pyruvate were tried on the same brain, one showed the difference and the other did not (Exps. 692, 693, Table I (*b*)). Some unknown variation in the state of the brains must influence the result. With this exception, the effects of the two poisons upon respiration are identical.

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[1] Rockefeller Fellow.

[2] Senior Demy, Magdalen College.

Table I. *Effect of poisons upon respiration of avitaminous brains.*

(*a*) *Effects of fluoride and iodoacetate upon oxygen uptake.* μl./g./hr.

Average respiration rates. January-July.

	No. of exps.	Substrate	Poison	Period (hrs.) 0–½	½–1	1–1½	1½–2
Normal	5	Lactate	—	2600	2210	1880	1580
		,,	Fluoride	1640	1230	810	550
	3	Lactate	—	2715	2305	1965	1690
		,,	IAA	1923	1013	533	275
Avit.	3	Lactate	—	1650	1280	980	800
		,,	Fluoride	1070	725	465	305
	3	Lactate	—	1730	1295	985	840
		,,	IAA	1285	755	435	280
	4	Pyruvate	—	1085	790	600	465
		,,	Fluoride	660	600	405	305
	4	Pyruvate	—	1465	1035	775	585
		,,	IAA	1035	535	320	185
	10	Lactate +	—	1450	1020	800	650
		pyruvate	IAA	1100	595	340	230

Summary of differences. μl./g./hr.

	Substrate	Poison	0–½	½–1	1–1½	1½–2	% resp. stable to poison			
Normal	Lactate	Fluroide	960	980	1070	1030	63	56	43	38
	,,	IAA	790	1290	1430	1420	71	44	27	16
Avit.	Lactate	Fluoride	580	555	515	495	65	57	47	38
	,,	IAA	445	540	550	560	74	58	44	33
	Pyruvate	Fluoride	425	190	195	160	68	76	68	66
	,,	IAA	430	500	455	400	71	52	42	32
	Lactate + pyruvate	IAA	350	425	460	420	76	59	43	35

(*b*) *Pyruvate—fluoride—IAA. September.*

Avit. Exp.	Substrate	Poison	0–½	½–1	1–1½	1½–2	Difference 1–1½
692	Lactate	—	2180	1835	1330	1000	
	Pyruvate	—	1710	1350	1030	830	
	,,	Fluoride	1125	850	530	350	500
	,,	IAA	1180	580	340	160	690
693	Lactate	—	1850	1280	925	720	
	Pyruvate	—	2140	1595	1370	1105	
	,,	Fluoride	1270	820	470	335	900
	,,	IAA	1595	850	475	280	895

(*c*) *Catatorulin effect—average values.*

Increase produced by vitamin in poisoned systems.

No. of exps.	Substrate	Value	0–½	½–1	1–1½	1½–2
2	Lactate	(L + F + V) − (L + F)	160	90	115	60 μl.
4	Pyruvate	(P + F + V) − (P + F)	160	200	140	140 μl.
10	Lactate − pyruvate	(LP + IAA + V) − (LP + IAA)	195	100	80	60 μl.
	% total vitamin effect, *i.e.* of (LP + V) − LP		46	20	15	10

Table I (*cont.*).

(*d*) *Influence of fluoride + IAA upon catatorulin effect.*

Exp.						
612	LP	1700	1365	1020	880	
	LPV	1980	1805	1240	1360	
	LPIAA	1700	980	380	170	
	LPIAAV	1840	1165	655	470	
	LPIAAF	1115	555	310	205	
	LPIAAVF	1255	720	320	245	
613	LPIAA	825	470	305	200	
	LPIAAV	1090	815	585	428	IAA. 0·12 mg./ml.
	LPIAAF	565	365	210	136	
	LPIAAVF	630	390	235	185	

In this and other Tables L = lactate; P = pyruvate; IAA = iodoacetic acid; F = fluoride; V = vitamin B_1.

Note. It is to be understood that in all cases medium consisted of Ringer phosphate-pyrophosphate with addition of lactate 0·03 M or pyruvate 0·02 M. LP = L 0·03 M, P 0·01 M.

The catatorulin effect (Table I (*c*)) is present but reduced to somewhat the same extent in each case; Exps. 612, 613 (Table I (*d*)) show that the poisons reinforce each other's action; both together eliminate the vitamin action.

Effect upon preformed pyruvate.

Avitaminous brain was allowed to form pyruvate by respiration for 2 hours in oxygen and lactate solution, at the end of which time additions of vitamin and tissue poisons were made. Pyruvate was estimated in most cases as bisulphite-binding substances [Clift and Cook, 1932] at the end of a further 1¼ hours. This was occasionally checked by Case's method.

The details of the experiment were as follows. Avitaminous cerebrum, minced warm, was distributed in five bottles containing Ringer phosphate and lactate 0·03 M. Pyruvate was formed by allowing respiration in O_2 for 2 hours from time of putting in the bath, the oxygen uptake being recorded for the last half hour, in order to check the values. At the end of this time, one bottle was removed as control and treated with 0·9 ml. trichloroacetic acid (25 %). The remaining four bottles were removed from the bath, one kept as control, one treated with vitamin (0·1 ml. containing 2γ), another the poison in 0·2 ml. and the last poison and vitamin; the bottles were quickly re-evacuated, filled with oxygen and returned to the bath. This operation need not occupy more than 7 minutes. Respiration was allowed to continue for 1 hr. 12 mins. after returning to the bath, the rate being calculated for the periods 12–42 mins. and 42–72 mins. No pyrophosphate was used in order to minimise the effects of the preparative stage (see preceding paper). At the end of 42 mins. the remaining bottles were removed from the bath and treated with trichloroacetic acid. Other details were as described by Peters and Thompson [1934].

It was thought better in this experiment to depart from the usual practice of using duplicate bottles for each point, and to use instead larger amounts of tissue from the cerebrum only, which is well known to be more homogeneous. Attempts to weigh exact amounts of brain "brei" introduce in themselves the qualitative errors of uneven cooling during the interval required for such weighing. We have therefore followed the usual practice of distributing the tissue by eye. This has not always been successful. We have therefore corrected for the tissue weights in the following way.

Variations in tissue weight for amounts of tissue over 100 mg. were found by Meiklejohn not to affect the oxygen uptake over 3-hour periods in the case of the cerebrum. This is contrary to the calculations of Passmore *et al.* [1933], who were able to correct for small amounts of tissue by the application of a formula. The difference between these two sets of results is not yet clear. The following diagram (Fig. 1) shows that oxygen uptake is independent of the amount of tissue for amounts between 140 and 250 mg. but the amount of pyruvic acid varies with tissue weight.

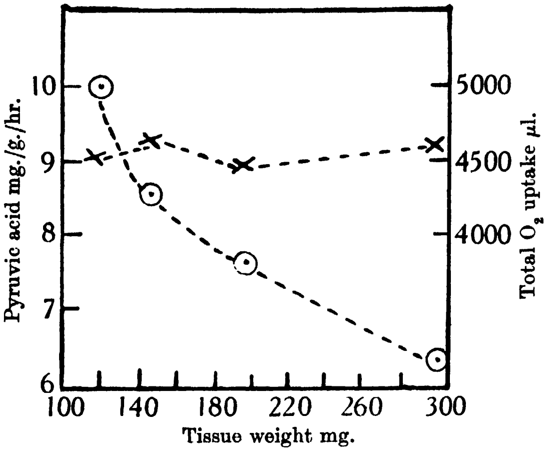

Fig. 1. ⊙ Pyruvic acid formed per tissue wt. × O_2 uptake per tissue wt.

Table II. *Pyruvic acid, poisons and the catatorulin effect.*

Typical experiments.

↓, substrate addition.

Oxygen uptake.

Exp. 678.

Hours ...	(a) 1½–2	↓	(b) 2¼–2¾	(c) 2¾–3¼
L	1195		815	600
LF	1180		710	420
LV	1110		990	1000
LFV	1120		880	650

Exp. 683.

Hours ...	(a) 1½–2	↓	(b) 2¼–2¾	(c) 2¾–3¼
L	710		590	275
LIAA	700		475	355
LV	700		700	580
LIAAV	700		625	415

Average decrease in respiration for (b) and (c) as compared with (a).

	(b) %	(c) %
L	-24	-50
LV	- 9	-19
LF	-42	-66
LFV	-21	-46

	(b) %	(c) %
L	-27	-55
LV	-10	-19
LIAA	-37	-55
LFV	-20	-33

Pyruvic acid. Pyruvate estimations corrected to weight of 160 mg. tissue weight along tangent to curve.

ml. $N/100$ I/g. (1 ml. = 0·44 mg. pyruvic acid).

Fluoride:

Exp.	Initial L	Final L	L+F	L+V	L+V+F
678	**6·19**	**9·8**	8·6	**7·2**	**7·44**
679	5·7	**8·3**	**6·2**	6·8	**5·7**
680	5·7	**7·9**	**6·9**	**4·9**	5·4
685	—	**9·7**	**8·4**	**8·0**	7·6
686	—	9·7	**8·5**	7·2	**7·2**
Average	5·9	9·1	7·7	6·0	6·7 ml. I/g.

Iodoacetate:

Exp.	Initial L	Final L	L+IAA.	L+V	L+V+IAA.
681	**5·9**	7·1	**8·1**	**6·0**	**7·3**
682	**9·0**	13·0	**11·3**	7·8	9·8
683	**7·4**	**9·2**	**8·9**	7·9	8·9
684	**5·7**	**7·7**	**8·1**	5·7	7·0
687	**7·3**	8·8	9·7	7·1	**8·5**
Average	7·1	9·2	9·2	6·9	8·3

Note. **In 682 and 683, L+V, L+V+IAA, also same wt.**

This is incidental evidence of the lack of correlation between O_2 uptake and pyruvic acid formation. The results given in Table II have been corrected for initial pyruvate (at end of 2 hours) to a basis of 160 mg. using the curve given in the figure. The conclusions which emerge from the averages are not however dependent upon this; in several cases tissue weights have been sufficiently close to require no correction. These are marked in heavy type.

Table II confirms the finding of Table I, that the O_2 uptake is reduced equally within the wide limits of error by the two poisons. The effect upon the pyruvate is strikingly different. It is decreased by fluoride and not affected by iodoacetate. In confirmation of Peters and Thompson, vitamin reduces the amount of pyruvate present. We must suppose that removal of pyruvic acid is already at a minimum in the avitaminous tissue in absence of vitamin so that no further accumulation takes place in presence of iodoacetate. So far as the origin and disappearance of pyruvic acid are concerned, the results are in good agreement with Meyerhof and Kiessling [1933]. As with the oxygen uptake, they do not give ground for the belief that the action of the vitamin is more concerned with iodoacetate than with fluoride, since no stress can be laid upon the variable results with pyruvate and fluoride. It must be noticed that in presence of vitamin, fluoride does not decrease significantly the amount of pyruvate formed.

Glutathione and added pyruvate.

Relatively more is known about the action of iodoacetic acid than about that of fluoride. We have therefore tried to analyse this further. The following is selected from the literature as to the action of iodoacetic acid.

1. Inhibition of lactic acid formation in muscle [Lundsgaard, 1930] and other tissues [Fisher, 1931].

2. Reduction of tissue respiration in muscle, which is partly restored by lactate addition [Meyerhof and Boyland, 1931]. Lactate improves contraction of muscle supplied with oxygen and poisoned with iodoacetate, and phosphagen synthesis [Mawson, 1932; 1933]; rather similar effects occur with nerve [Chang and Gerard, 1933].

3. Low concentrations interfere with the catabolism of glycogen (A), Meyerhof and Kiessling [1933], and with the action of glyoxalase in tissue sections (B) [Dickens, 1933; Quastel and Wheatley, 1932. *Cf.* Dudley, 1931].

4. Higher concentrations prevent the interaction of α-glycerophosphate and pyruvic acid (C). They also inhibit the action of glyoxalase in some brei preparations, the inhibition being believed to be due to the removal of glutathione [Dickens, 1933].

Hence at least three enzyme systems A, B, C, must be poisoned. Our present effect might be due to B or C. If to B alone it should be restored by adding glutathione.

In a series of seven experiments the effect of glutathione with and without vitamin upon pyruvate was studied in presence of iodoacetate. In order to ensure the presence of adequate pyruvate, a small amount of this was added to the medium, which also contained pyrophosphate to enhance vitamin effects. Some of the O_2 uptakes are summarised in Table I. In Table III, 1, 2, 3 are given only the figures relating to glutathione and the values for pyruvate.

In order to observe the action properly, the tissue should be kept in contact with the reagents for a standard time before adding the glutathione. In practice the tissue was allowed to stand for 3 minutes after division with the crusher, iodoacetate was then added, after an interval of 2 minutes glutathione and after a further interval vitamin.

Table III. *IAA, glutathione and pyruvate.*

Medium (M) = lactate-pyruvate-pyrophosphate. G = glutathione, 0·0021 *M*. V = vitamin 2γ.

1. *Oxygen uptake.* Average level for 7 experiments.

	Period (hours)			
	0–½	½–1	1–1½	1½–2
M.IAA	1100	595	340	230
M.G.IAA	1200	760	570	390
Diff.	100	165	230	160
M.IAA.V	1295	695	420	290
M.IAA.V.G	1450	1080	795	620
Diff.	155	385	375	330

2. Effect of glutathione and iodoacetic acid on the removal of added pyruvic acid: expressed as mg. pyruvic acid disappearing per g. of tissue.

Exp.	M	MV	MIAA	MIAAV	MIAAG	MIAAGV
600	−0·56	−2·73	−0·22	−1·63	−0·41	−1·97
601	+0·09	−3·14	+1·03	−2·92	−0·21	−1·91
604	−0·78	−4·66	+1·00	−0·10	+0·32	+2·77
605	−2·24	−4·41	−0·53	−1·12	−1·62	−4·16
607	−1·09	−2·51	+0·79	+0·13	−0·38	−2·43
608	−0·97	−3·14	−0·10	−1·43	—	—
612	−2·70	−4·47	−0·35	−1·47	—	—

3. Effect of glutathione and iodoacetic acid on the vitamin effect.

In 3, M≡MV − M, etc.

(A) Extra disappearance of pyruvic acid due to vitamin action.

Exp.	M	MIAA	MIAAG
600	2·17	1·41	1·56
601	3·23	3·95	1·70
604	3·87	1·10	3·09
605	2·17	0·59	2·54
607	1·42	0·66	2·06
Average	2·57	1·54	2·19

(B) Extra O_2 uptake due to vitamin action. (μl./g./hr.)

		Period (hours)			
Exp.	Medium	0–½	½–1	1–1½	1½–2
600	M	320	525	590	845
	MIAA	(440)	137	−181	−47
	MIAAG	205	408	123	248
601	M	325	385	505	520
	MIAA	65	−65	10	35
	MIAAG	220	265	227	190
602	M	545	735	713	705
	MIAA	115	80	−7	72
	MIAAG	340	340	205	240
603	M	85	345	410	465
	MIAA	(390)	−25	−102	15
	MIAAG	405	475	350	365
604	M	465	400	705	690
	MIAA	−295	105	54	83
	MIAAG	135	100	97	45
605	M	555	640	615	805
	MIAA	260	180	199	55
	MIAAG	325	400	415	422
607	M	335	325	490	495
	MIAA	90	−55	180	−25
	MIAAG	130	200	230	136
Averages	M	375	479	575	646
,,	MIAA	47	51	22	27
,,	MIAAG	251	318	235	235
Actual restoration with G (MIAAG–MIAA)		204	267	213	208
% restoration with G		54·5	56	37	32

Note. In Exp. 600, the pyruvic acid was estimated by the isolation of the 2 : 4-dinitrophenylhydrazone, in all other experiments by bisulphite-iodine titrations.

The increases seen in MIAA 601, 604 and 607 are probably not experimental errors, but due to the formation of pyruvate by the tissue (compare Table II).

In these experiments addition of glutathione restored the vitamin effect to some 30–50 %. Part too of the pyruvate seems to be removed again upon addition of glutathione, but this cannot be certainly concluded owing to the large experimental error. The dubiety of this result makes interpretation difficult. Iodoacetate is known to interact slowly, and it is not known how far glutathione will diffuse into these tissue systems, though in general it is freely diffusible. We think it probable that the glutathione is merely combining with the excess of iodoacetate, thereby preventing further action; if the effect were to restore a missing catalytic effect of glutathione we should have expected the recovery with the added substance to be more nearly maximum. Hence we do not think that glutathione is directly concerned with the vitamin effect; in this we have the support of earlier experiments with glutathione alone. So far judgment from the respiration experiments of Peters and Sinclair [1933] excludes interaction with α-glycerophosphate. Hence both A and C seem also excluded and we are left with the inhibition of some unknown metabolic link.

Table IV. *Delayed substrate experiments with poisons.*

Each experiment made upon uniform sample of brain tissue.

Exp. No.	Initial addition	Addition at ↓	1¾–2¼		Resp. period from start of experiment (hours)				
					2½–2¾	2¾–3¼	3¼–3¾	3¾–4¼	4¼4¾
Fluoride:									
641	O	LPppV	420	↓	450	390	350	330	—
	O	O	470	↓	310	230	230	170	—
	V	LPpp	545	↓	520	625	600	535	—
	V	O	640	↓	455	440	340	280	—
	V	LPppF	590	↓	580	520	340	280	—
	VF	LPpp	220	↓	300	175	130	90	—
645	V	LF	700	↓	855	885	845	800	—
	O	LFV	500	↓	570	575	625	615	—
	F	LV	190	↓	200	160	160	120	—
	F	V	170	↓	90	60	150	70	—
	FV	L	240	↓	270	220	170	174	—
	FV	O	260	↓	230	120	150	100	—
646	O	PFV	550	↓	445	435	290	190	—
	V	PF	625	↓	680	740	480	270	—
	F	PV	190	↓	195	210	185	65	—
	F	V	215	↓	240	160	85	145	—
	FV	P	225	↓	250	190	215	70	—
	FV	O	220	↓	210	160	150	65	—
IAA:									
642	ppV	LP	660	↓	1170	995	930	935	—
	ppV	O	710	↓	530	495	410	310	—
	pp	LPV	500	↓	770	640	540	635	—
	pp	O	500	↓	480	355	310	290	—
	ppV	LPIAA	575	↓	935	725	460	340	—
	ppV	IAA	605	↓	485	340	250	215	—
	ppIAA	LP	90	↓	300	100	115	85	—
	ppVIAA	O	115	↓	110	40	75	85	—
708	pp	LV	570	↓	870	700	730	780	655
	ppV	L	760	↓	1250	1090	1105	1035	—
	ppIAA	LV	225	↓	325	170	95	135	25
	ppV	L	285	↓	275	230	165	125	90
	pp	LVIAA	625	↓	940	800	640	495	320
	ppV	LIAA	760	↓	1325	1135	800	580	385

O = Ringer-phosphate; V = O + vitamin; pp = pyrophosphate. Pyruvate (P) approx. in Exps. 646, 0·02 *M*; in Exps. 641, 642, 0·007 *M*.

Delayed substrate experiments (Table IV).

There is a component of the preparative stage blocked by the action of the poisons. In experiments made by the delayed substrate technique (see preceding paper), poison in the preparative stage inhibits subsequent rise of oxygen uptake upon addition of lactate. The vitamin effect is eliminated. Poison added at the same time as substrate only acts slowly and does not inhibit the initial rise. We interpret this to mean that the poison acts upon the conversion of X into Y, but not the interaction of Y with substrate[1]. The later reduction of respiration of the tissue treated with lactate and poison at the same time is consistent with this view; it would be expected that after respiration for an hour with substrate, there would be a component of the respiration supplied by further formation of Y. The application of these results to the method of Quastel and Wheatley suggests that narcotics as well as iodoacetic acid will probably be acting upon the preparative stage.

It is to be noted that even after 2 hours of falling residual respiration, the capacity for forming pyruvic acid is not substantially diminished. Table V shows the few experiments made upon this question with avitaminous cerebrum. They suffice to indicate the rapid increase of pyruvate upon addition of lactate and again show the reduction in presence of vitamin. It will be recalled that there are reasons for thinking that pyruvate does not arise directly from lactate [Peters and Thompson, 1934].

Table V. *Formation of pyruvate in delayed substrate experiment.*

Bisulphite-binding substances present after respiration for periods indicated. ml. $N/100$ I/g. tissue (avitaminous cerebrum).

Exp.	RP After 2 hrs.		RP Hours +¼	RP Hours +¾	L Hours +¼	L Hours +¾	L +1 hr. 10 mins.	LV Hours +¼	LV Hours +¾	LV +1 hr. 10 mins.
671	—	↓	2·6	2·2	3·5	4·5	—	—	—	—
673	—	↓	—	—	3·4	4·0	—	2·5	2·6	—
675	1·6	↓	—	—	3·6	—	5·5	3·3	—	3·3
676	2·3	↓	—	—	3·8	5·4	—	3·8	3·6	—
Average	2·0	↓	2·6	2·2	3·6	4·6	—	3·2	3·1	—

RP = Ringer phosphate only. At arrow (end of 2 hours) addition of L or LV or nil made as indicated and respiration continued for additional periods.

DISCUSSION.

Catatorulin effect. The catatorulin effect is inhibited by action upon the preparatory stages equally by the two poisons, irrespective of the effect upon pyruvate. This reinforces the view that the action of vitamin upon pyruvate is secondary. It seems to be a generalisation that poisons influencing catatorulin action affect phosphorylation stages. This is even true for phloridzin [Lundsgaard, 1933]. With 3 mg./ml. in three experiments the "vitamin effect" was almost eliminated. There is no evidence that any of these poisons is specific for vitamin B_1. Iodoacetate destroys vitamin B_1 activity upon warming at p_H 7·3 in Ringer phosphate for 12 mins., but a short period of standing at room temperature hardly influences it.

Embden-Meyerhof scheme. This is followed to the extent that fluoride and iodoacetate influence appearance and disappearance of pyruvate as in the scheme. According to Johnson (in press)[1], the scheme cannot be applied so far as α-glycerophosphate is concerned.

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[1] See hypothesis in the previous paper.

Lactic acid. Chang and Gerard [1933] have found that with lactate present the fall in respiration of frog nerve is not so marked at first as subsequently in iodoacetate solutions. The figures of Table I bear this out for pigeon's brain. It is well explained by the hypothesis here advanced if iodoacetate poisons production of *Y*.

SUMMARY.

1. The actions of some poisons upon the avitaminous pigeon's brain tissue have been further investigated. Fluoride and iodoacetate reduce O_2 uptake equally. Each largely reduces the catatorulin effect (extra O_2 uptake in presence of added vitamin B_1): both together eliminate this.

2. The effect upon pyruvate formation by avitaminous brain is different; iodoacetate leads to increased accumulation and fluoride the reverse. So far only as pyruvate is concerned, this is consistent with the finding of Meyerhof and Kiessling for muscle extracts.

3. Glutathione only produces partial restoration of the vitamin effect in presence of iodoacetate.

4. Disappearance of pyruvate in presence of vitamin B_1 is an indirect result of some other change. These poisons influence a preparatory stage for oxidation.

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[1] Personal communication.

REFERENCES.

Chang and Gerard (1933). *Amer. J. Physiol.* **104**, 291.
Clift and Cook (1932). *Biochem. J.* **26**, 1788.
Dickens (1933). *Biochem. J.* **27**, 1141.
Dudley (1931). *Biochem. J.* **25**, 439.
Fisher (1931). *Biochem. J.* **25**, 1410.
Lundsgaard (1930). *Biochem. Z.* **217**, 162.
—— (1933). *Biochem. Z.* **264**, 209.
Mawson (1932). *J. Physiol.* **75**, 201.
—— (1933). *J. Physiol.* **78**, 295.
Meiklejohn, Passmore and Peters (1932). *Biochem. J.* **26**, 1872.
Meyerhof and Boyland (1931). *Biochem. Z.* **237**, 406.
Meyerhof and Kiessling (1933). *Biochem. Z.* **264**, 40.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Peters and Thompson (1934). *Biochem. J.* **28**, 916.
—— and Sinclair (1933). *Biochem. J.* **27**, 1910.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 269.

IX. STUDIES ON THE VITREOUS BODY. I.

By WILLIAM STEWART DUKE-ELDER,
EDWARD BLAIR ROBERTSON AND HUGH DAVSON.

*From the Department of Physiology and Biochemistry,
University College, London.*

(*Received November 2nd, 1934.*)

The aim of the studies detailed below was to elucidate the phenomenon of the rise of intra-ocular pressure associated with chronic glaucoma (*glaucoma simplex*).

The facts of the shallow anterior chamber, the pushing forward of the lens and iris, the thinning of the choroid and the compression of the retina, combined with the freedom of the iris in numerous cases of the disease, lead to the conclusion that a swelling of the vitreous body is most probably the determining factor. The view of Heesch [1926, 1] that the increase in pressure might be due to negative osmosis across the scleral membrane is contradicted by the fact of the compression of the sclera to less than its normal thickness. Furthermore any theory postulating that the increased pressure is due to the failure of elimination of fluid from the anterior chamber is unable to account for one of the most obvious symptoms of the disease, *viz.* the shallow anterior chamber.

The vitreous body is a true gel showing an ultra-microscopic fibrillar structure [Baurmann, 1923; 1926; 1932; Heesch, 1926, 2; Duke-Elder, 1929; Goedbloed, 1934] with the characteristic vapour pressure isotherm of a gel formed from a lyophilic colloid [Duke-Elder, 1930], and typical elastic properties [Robertson and Duke-Elder, 1933]. It contains a protein called "residual protein" [Duke-Elder, 1930] in a concentration of 0·025 % which is responsible for its gel nature.

The combined isolated proteins of the vitreous body show degrees of swelling depending on the p_H of the swelling medium rising from a minimum at about p_H 4·5 to a maximum at about p_H 8·0 [Duke-Elder, 1930]. Large variations in the volume of the vitreous body are achieved by placing it in solutions of different p_H [Baurmann, 1924; Duke-Elder, 1930], but owing to the crudeness of the technique employed any deductions drawn from these experiments must be treated with caution. It is true that Redslob and Reiss [1928] found considerable variations in the total volume of an intact eye in a modified dilatometer on varying the p_H. Two maxima at p_H 8·1 and 8·7 were obtained. Since however the sclera is known to increase in volume with varying p_H these results do not allow of any conclusions as to the behaviour of the vitreous body to be made. On the other hand, Löbeck [1929], using the isolated vitreous body, failed to record any increase in volume on the alkaline side of normal p_H.

So far there is no conclusive evidence that the vitreous body, *in vivo*, is capable of increasing its volume on altering the p_H of its surrounding medium. Glaucoma however is not associated with any detectable abnormality in the p_H of the blood [see *e.g.* Schmerl, 1928], so that experiments on the variation of this factor are only of interest to the extent that they show that the vitreous body is capable of modification of its volume in response to a changed environment.

A factor in the swelling of proteins often more potent than the p_H is the presence of specific ions, *e.g.* I^- or CNS^-, or the so-called hydrotropic substances of Neuberg [1916]. The recent work of Katz [1933] on the swelling of starch and gelatin has shown that substances such as the sodium and potassium salts of long-chain fatty acids, and in general dipolar substances with both hydrophilic and hydrophobic groups in their molecules, will increase the rate of swelling to an enormous extent. Since the increased swelling so produced is due to adsorption on to the micelles of the colloid, it is obvious that these substances may be effective in low concentrations, and hence it is not impossible that they are effective in the causation of glaucoma. The occurrence of abnormal quantities of substances of a soapy nature in the eye in advanced glaucoma and similar degenerative conditions of the eye lends support to this view. It may be remarked here that Baurmann and Thiessen [1922] noted that KCNS delayed the occurrence of "age-changes" in the vitreous body, and Goedbloed [1934] observed ultra-microscopic changes in it after treatment with KCNS solutions for 24 hours which he interpreted as due to increased hydration of the micelles.

In this paper the results of experiments are described which are designed to show whether substances such as sodium oleate and sodium laurate in aqueous solution may affect the volume of the vitreous body. A full discussion of the possibilities that a gel, in such a state of dilution as is the vitreous body, may exhibit an increase in volume or hydration even under the most favourable conditions, will be left to a succeeding communication when it is hoped that the results of experiments on other swollen gels, at present under way, will have given us sufficient information to allow of a more than tentative decision to be made.

EXPERIMENTAL.

The apparatus of Schmidt [1931], used for the determination of the water-uptake of powders, was modified to suit the peculiarities of the vitreous body (see Fig. 1). The tap, T_1, allows connections to be made between the reservoir, R, and the capillary, C; between R and the chamber, P; between P and C, and between R, P and C. The vitreous body is placed in the chamber, P, which is separated from the fluid in the rest of the apparatus by a sintered glass disc, S, and which has a mark on it showing the level of the tap, T_1. Any uptake of water is measured by the movement of the meniscus in the capillary. The procedure is to fill the reservoir with the given solution and make connection, with T_2 open, to P and C until fluid flows through T_2 when the latter is closed and connection is made between R and C alone until the latter is half filled. T_1 is then half-turned so that no connections are made, and the vitreous body of an ox is placed in P to just above the mark. When the tap T_1 is turned to make connection between P and C the meniscus will move along the capillary to the right due to the slight excess of hydrostatic pressure in P. To counteract this the whole apparatus is tilted slightly as the tap is turned so that the meniscus stops after it has moved a few inches. The tilting must not be so steep that the meniscus starts to move backwards, otherwise water will enter P and the experiment must be started again. Solutions of 0·9 % NaCl buffered with phosphate to p_H 7·8 and 8·0, and solutions of 1 % sodium oleate and laurate were used.

Results.

In all cases no water-uptake was observed in 24 hours.

As will be appreciated, the apparatus described determines whether the gel takes up water, but not whether a volume increase occurs, although the one process is always associated with the other. Küntzel [1929] has divided the swelling process into two parts, one due to water-uptake and the other due to the mutual repulsion of charged micelles. Neville and Theis [1930] have shown that a maximum of swelling need not necessarily correspond with a maximum of water-uptake. In view of the possible independence of the two processes at certain stages of swelling it was thought that there was just a chance that the vitreous body might alter its volume by a re-orientation of the charged micelles, produced by the adsorption of a polar compound, without necessarily taking up water. To put this to the test, the apparatus shown in Fig. 2 was devised.

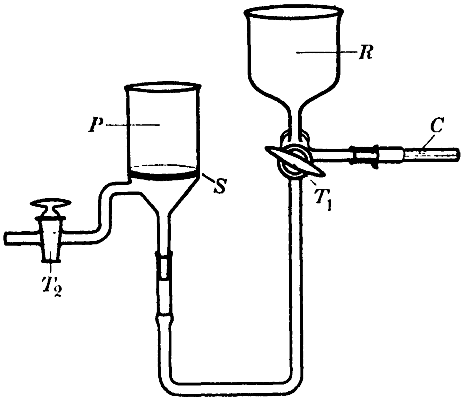

Fig. 1.

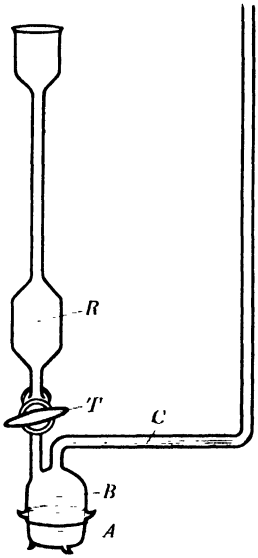

Fig. 2.

The hollow hemisphere, A, fits into the dome, B, by a ground joint. B is connected to a capillary, C, and a reservoir, R. A is filled with vitreous and fitted into B. R is filled with a saturated solution of sodium laurate in amyl alcohol dried with sodium sulphate. The whole is allowed to equilibrate in a thermostat at 25° (constant to 0·002°). When thermal equilibrium has been established the tap T is turned and the solution is allowed to fill up B and run along the capillary tube. The tap is turned off and the position of the meniscus fixed on the cross-wires of a telescope. Any increase in the volume of the vitreous would be registered by a movement of the meniscus to the right. A control was run using pure dry amyl alcohol.

Repeated experiments failed to show an increase of volume; in general a slight contraction in both control and test experiments was observed.

Discussion.

The absence of any water-uptake near p_H 8 in the first experiment is in agreement with the findings of Löbeck; the experiments with soaps show that under more extreme conditions than that of changed p_H the vitreous body does not increase in volume within 24 hours.

These results however can hardly be treated as absolutely demonstrating that the vitreous body is incapable of increasing in volume in *glaucoma simplex*,

as the increase in intra-ocular pressure associated with this disease is slow, frequently taking years to reach its maximum value. Furthermore, there seems to be a definite difference in the behaviour of the vitreous body according as it is *in vitro* or *in vivo*. For example, in the latter state it is capable of withstanding unbalanced stresses of quite a high order, whereas *in vitro* the slightest unbalanced stress tends towards liquefaction. Considerations such as these make it difficult, in the present state of our knowledge, to reason from the behaviour of the vitreous body *in vitro* as to its probable behaviour *in vivo*.

We have pleasure in recording our indebtedness to Profs. J. C. Drummond and H. Freundlich for their sustained interest and criticism, and to the Medical Research Council for defraying the expenses of this research and for personal grants to two of us (E. B. R. and H. D.).

REFERENCES.

Baurmann (1923). *Arch. Ophthal.* **111**, 353.
—— (1924). *Arch. Ophthal.* **114**, 280.
—— (1926). *Arch. Ophthal.* **117**, 304.
—— (1932). *Ber. Versamml. deutsch. ophthal. Ges.* **49**, 44.
—— and Thiessen (1922). *Nachr. Götting. Ges.* 125.
Duke-Elder (1929). *J. Physiol.* **68**, 155.
—— (1930). *Brit. J. Ophthal. Mon.* Suppl. IV.
Goedbloed (1934). *Arch. Ophthal.* **132**, 323.
Heesch (1926, 1). *Arch. Augenheilk.* **97**, 546.
—— (1926, 2). *Klin. Monatsbl. Augenheilk.* **77**, 206.
Katz (1933). *Biochem. Z.* **257**, 385, 397; **259**, 76, 191, 223; **261**, 15, 47, 433; **262**, 355; **263**, 323, 421.
Küntzel (1929). *Biochem. Z.* **209**, 326.
Löbeck (1929). *Arch. Ophthal.* **122**, 668.
Neuberg (1916). *Biochem. Z.* **76**, 107.
Neville and Theis (1930). *Coll. Symp. Mon.* **7**, 41.
Redslob and Reiss (1928). *Compt. Rend. Soc. Biol.* **2**, 219.
Robertson and Duke-Elder (1933). *Proc. Roy. Soc. Lond.* B **112**, 215.
Schmerl (1928). *Arch. Augenheilk.* **48**, 565.
Schmidt (1931). *Z. physikal. Chem.*, Bodenstein Festband, 333.

X. THE CHROMOGEN OF MELANURIA.

By LOUIS LINNELL and HENRY STANLEY RAPER.

From the Department of Physiology, University of Manchester.

(*Received November 12th, 1934.*)

THE course of a melanotic sarcoma is sometimes marked by the excretion in the urine of a chromogen which readily oxidises on exposure to air to the black pigment, melanin. It has been shown by Raper [1927] that in the formation of melanin from tyrosine or 3 : 4-dihydroxyphenylalanine one intermediate product is 5 : 6-dihydroxyindole: a colourless material which rapidly oxidises to melanin in the air. It seemed possible that the chromogen might be this same indole derivative, or some closely related substance, and this view has been rendered more probable by the work of Bloch and Schaaf [1925] who showed that the production of melanin in the skin is due to the action of an enzyme, dopa-oxidase, on 3 : 4-dihydroxyphenylalanine. It has also been shown that tyrosinase is present in the skin of certain black rabbits [Onslow, 1915; Pugh, 1933] and in melanotic tumours [Gessard, 1903; De Coulon, 1920]. Clearly, if it could be demonstrated that the chromogen of melanuria is related to 5 : 6-dihydroxyindole, this would be good evidence for the view that in the higher animals melanin formation is due to the same series of changes that occur in the lower animals and plants, in which tyrosinase reacts with a suitable substrate such as tyrosine, 3 : 4-dihydroxyphenylalanine, or tyramine, 3 : 4-dihydroxyphenylethylamine.

Eppinger [1910] examined the urine from a case of melanotic sarcoma and concluded that two melanogens were present: one soluble, the other insoluble, in ether. He was able to isolate an ether-insoluble crystalline substance which appeared to be the amide of an ethereal sulphate of *N*-methylhydroxypyrrolidinecarboxylic acid. The product showed some of the properties associated with a melanogen and was considered by Eppinger to be the ether-insoluble chromogen, although in the light of recent work it seems most unlikely that a substance of the constitution suggested could be a precursor of melanin. The ether-soluble chromogenic material was further studied by Feigl and Querner [1917] who separated it into three fractions by precipitation with light petroleum and cadmium chloride. In this connection it may be noted that Thannhauser and Weiss [1922] isolated from melanotic urine an ether-soluble catechol derivative which they suggested was catecholacetic acid.

The view that tryptophan was the parent substance of the melanogen was advanced by Eppinger on his discovery that the administration of this amino-acid to the patient caused the excretion of the chromogen, as estimated by the Thormählen reaction, to rise considerably.

Our own experiments which are described below were planned to determine whether the administration to animals of the indole derivatives produced by the action of tyrosinase on tyrosine would lead to a condition of melanuria such as is encountered in melanotic sarcoma, and if this were so, whether the melanogens in the two cases had similar properties. We have also synthesised the ethereal disulphate of 5 : 6-dihydroxyindole in order to compare its reactions with those of the melanogen, since it seemed possible that this substance might be excreted in the urine if the dihydroxyindole either arose as a metabolic product or was administered directly.

EXPERIMENTAL.

I. *The melanogen from human urine.*

Through the courtesy of Dr G. A. Harrison, of St Bartholomew's Hospital, London, who kindly furnished the melanotic urine and crude melanogen precipitate, we were able to study the chromogen excreted in human melanuria.

On exposure to air the urine slowly blackened from the surface downwards and eventually a deposit of melanin appeared. The blackening could be prevented by keeping the urine in an oxygen-free atmosphere or by the addition of a small quantity of sulphurous acid. The urine exhibited all the characteristic melanogen reactions, of which the following are the more important.

The hydrochloric acid reaction. On heating the urine with concentrated hydrochloric acid it rapidly becomes dark brown or black.

The ferric chloride reaction. The addition of a few drops of ferric chloride solution causes the urine to turn grey.

The ferricyanide reaction. Dilute solutions of ferric chloride and of potassium ferricyanide are mixed and acidified with hydrochloric acid. On the addition of a few drops of urine there is a copious formation of Prussian blue. This reaction is given to some extent by normal urine.

The Ehrlich reaction. To 1 ml. urine are added 2–3 drops of a 5 % solution of *p*-dimethylaminobenzaldehyde in alcohol and 5 ml. alcohol. On the addition of 0·5 ml. concentrated hydrochloric acid a violet colour appears either in the cold or on warming. The addition of a few drops of very dilute sodium nitrite solution changes the colour to deep purple.

The Thormählen reaction. To 1 ml. urine are added a few drops of a dilute solution of sodium nitroprusside, followed by a little caustic soda. A violet-red colour is immediately produced. The addition of excess acetic acid now changes the colour to pure blue.

Both the Ehrlich and the Thormählen reactions are very delicate, although the former is considerably the more sensitive of the two. It is to be noted that both these reactions are characteristic of indole and some of its derivatives.

The urine was precipitated with lead acetate in the presence of acetic acid in order to remove phosphates and sulphates. The melanogen was then completely precipitated from the filtrate by the addition of ammoniacal lead acetate. The yield of dried melanogen precipitate was about 70 g. from 10 litres urine. It was yellowish white in colour and did not darken on keeping. 5 g. of this precipitate were ground up with 100 ml. distilled water, and a current of carbon dioxide was passed through the suspension with frequent shaking. After two hours the suspension was filtered and the precipitate washed. The filtrate was pale yellow in colour and gave intense Ehrlich and Thormählen reactions. The precipitate was again ground up with 100 ml. water and treated with carbon dioxide, the filtration and decomposition with carbon dioxide being repeated until the filtrate gave only very weak Ehrlich and Thormählen reactions.

The mixed filtrates were saturated with hydrogen sulphide to remove lead from solution, and after filtration, the liquid was concentrated to small bulk by evaporation under reduced pressure in a stream of hydrogen. The syrup so obtained was brought to dryness in a vacuum-desiccator. A brown resin remained: yield: 1·03 g. A repeat experiment gave a yield of 1·14 g.

The resin was rubbed up with methyl alcohol, in which it partially dissolved. The methyl alcoholic solution was filtered and brought to dryness in a vacuum-desiccator. This gave residue "A." The insoluble material was washed with methyl alcohol and dried in a vacuum-desiccator, giving residue "B."

The lead precipitate remaining from the carbon dioxide treatment was re-suspended in water and decomposed with hydrogen sulphide. After filtration, the liquid was concentrated to small bulk by evaporation under reduced pressure in a stream of hydrogen and finally brought to dryness in a vacuum-desiccator, giving residue "C."

Residue "A" was a brown semi-solid resin which would not crystallise; yield: 0·66 g; repeat: 0·88 g. It was further purified by redissolving in methyl alcohol, cooling the solution on ice, filtering from a small quantity of residue and again evaporating to dryness. The substance gave intense Thormählen and Ehrlich reactions and blackened when warmed with hydrochloric acid. On the addition of ether it was precipitated from methyl alcoholic solution in the form of very hygroscopic white flocks which rapidly changed to a black oil. Tollens's naphthoresorcinol test indicated the presence of a trace of glycuronate.

The material contained sulphur, nitrogen and a trace of halogen, but no phosphorus. On analysis by the method of Benedict, as modified by Denis (*i.e.* ignition with copper nitrate) the total sulphur content was found to be 2·29 %. The amount of sulphur present in the form of ethereal sulphate was found to be 1·68 %; repeat estimation: 1·60 %.

An attempt was made to effect a further purification of the melanogen by precipitating it as its mercury compound. Previous investigators have used Hopkins's reagent, 10 % mercuric sulphate in 10 % sulphuric acid, for this purpose. In view of the instability of the chromogen towards mineral acid it was thought that the sulphuric acid in Hopkins's reagent might have a deleterious effect. For this reason, mercuric acetate was used in place of mercuric sulphate.

0·52 g. material "A" was dissolved in 20 ml. distilled water and completely precipitated by the addition of a 2 % solution of mercuric acetate in 2½ % acetic acid (20 ml. of reagent required). The yellowish white precipitate was filtered off on paper pulp, washed, suspended in water and decomposed with hydrogen sulphide. After filtration, the liquid was concentrated to small bulk by distillation under reduced pressure in a stream of hydrogen and finally taken to dryness in a vacuum-desiccator. This gave residue "D." The filtrate remaining after the removal of the precipitate was saturated with hydrogen sulphide and, after filtration from mercuric sulphide, brought to dryness as before, giving residue "E."

The substance "D" was a dark brown solid which easily powdered on being rubbed; yield: 0·18 g. The powdered material showed no crystalline form: it was very hygroscopic and readily soluble in water, giving a brown solution which gave a fair Ehrlich reaction, good ferric chloride and hydrochloric acid reactions, but no Thormählen reaction. The solution was very acid to litmus. The solid was also soluble in methyl and ethyl alcohols, but not in ether, benzene, light petroleum, acetone or chloroform. The amount of sulphur present in the form of ethereal sulphate was found to be 2·86 %. The dark colour of the product, the weakness of the Ehrlich and absence of the Thormählen reactions suggested that a considerable decomposition of the melanogen occurred in the mercuric acetate precipitation process, and for this the acidity of the solution was, perhaps, responsible.

The residue "E" was a pale yellow viscid syrup which could not be crystallised; yield: 0·24 g. Amount of sulphur present as ethereal sulphate: 0·99 %.

Since the Ehrlich and Thormählen reactions were absent and the ferric chloride reaction weak, it was concluded that the substance was not a melanogen.

The material "B" was a pale pink powdery solid which was not hygroscopic; yield: 0·37 g.; repeat: 0·26 g. It was readily soluble in water and in dilute acids and alkalis, insoluble in methyl and ethyl alcohols, benzene, light petroleum and acetone. The substance was purified by dissolving in hot dilute ethyl alcohol, filtering rapidly and cooling. An emulsion formed which eventually solidified into flocks. The product was almost white in colour, entirely amorphous in appearance, gave a weak Ehrlich, but no Thormählen reaction, and no coloration with neutral or acidified ferric chloride. The pale pink coloration of the solid was probably due to a trace of melanogen and this would account for the positive Ehrlich reaction (which is more sensitive than the other melanogen tests). In aqueous solution it was acid to litmus but not to Congo red. On heating, it charred with a peculiar odour, melted and finally burnt completely away. It contained sulphur and nitrogen but no phosphorus. From the ready solubility in water it followed that the substance could not be cystine: whilst the absence of a reaction with sodium nitroprusside after boiling with caustic soda proved that it was no derivative of cysteine. Since it gave no coloration with ferric chloride it could not be a melanogenic substance. The presence of a sulphate group, ethereal or otherwise, could not be detected, so it was concluded that the sulphur existed as so-called "neutral sulphur." The only compounds containing neutral sulphur which have hitherto been found in urine and which resemble the substance "B" are antoxyproteic, oxyproteic, alloxyproteic and uroferric acids. Their principal reactions have been described by Bondzyński and Panek [1902] and by Bondzyński *et al.* [1905]. On applying these reactions to "B," it was clear that it was not a proteic acid. Its further examination was deferred.

The substance "C" was a yellowish white amorphous material; yield: 0·07 g. It was almost insoluble in water, but dissolved in dilute caustic soda, from which it was precipitated by sulphuric acid. Its suspension in water was faintly acid to litmus and did not decolorise bromine water. No nitrogen was detected, but traces of sulphur and halogen were present. The presence of a trace of melanogen probably accounted for the appearance of weak Thormählen, Ehrlich and ferric chloride reactions. As the material was evidently not a chromogen it was not further investigated.

II. *The production of melanuria by the administration of 5 : 6-dihydroxyindole.*

The method of Raper [1927] was used for the preparation of a solution of 5 : 6-dihydroxyindole from tyrosine by the action of tyrosinase. The solution obtained on the oxidation of 2 g. tyrosine was concentrated to about 10 ml. by evaporation under reduced pressure in a current of hydrogen, filtered from unchanged tyrosine, and its neutrality ensured towards Congo red. The liquid was then immediately injected subcutaneously into a rabbit. The urine excreted thereafter for about 30 hours had the typical properties of a melanotic urine: after the lapse of this period the excretion of a normal urine was resumed.

The colour of the "melanotic" rabbit urine was normal when fresh, but on standing, the urine blackened from the surface downwards. It gave a good hydrochloric acid reaction and very strong ferric chloride, Thormählen and Ehrlich reactions. On the other hand, there was no alteration in appearance on the addition of caustic soda, even on boiling. This immediately proved that no 5 : 6-dihydroxyindole was excreted unchanged, for this substance is very sensitive to alkali and, even very dilute solutions of it blacken when caustic soda is added. The urine also gave a good positive reaction on applying Tollens's test for glycuronates; normal rabbit urine gave this reaction but not so well.

The separation process previously used with the human melanotic urine was applied, with a small modification, to the rabbit urine. To the fresh urine was added sufficient dilute acetic acid to give an acid reaction to litmus, followed by an excess of 20 % lead acetate. The acid lead precipitate was filtered off and washed, and the filtrate and washings were mixed and rendered alkaline by the addition of dilute ammonia, whereupon a yellow precipitate was thrown down. This was filtered off and washed. A sample of each precipitate was suspended in water, decomposed with hydrogen sulphide, the solution filtered and a current of air passed to remove hydrogen sulphide.

The solution from the basic lead precipitate gave strong Thormählen, Ehrlich and ferric chloride reactions, whilst neither the solution from the acid lead precipitate nor the filtrate remaining from the two precipitations gave these reactions. Evidently the basic lead precipitate contained the whole of the chromogen. It was dried in a vacuum-desiccator to obtain it in a stable condition. By the injection into rabbits of the 5 : 6-dihydroxyindole prepared by the action of tyrosinase on 12 g. tyrosine, a total yield of 19·33 g. dry melanogen precipitate was secured from a bulk of 1188 ml. urine.

5 g. dried precipitate were ground up with 150 ml. distilled water, and the suspension was saturated with carbon dioxide and mechanically shaken for 4 hours. The suspension was then filtered, the precipitate resuspended in water and the saturation with carbon dioxide repeated until the filtrates ceased to give the melanogen reactions. Lead was removed from the mixed filtrates by the passage of hydrogen sulphide in the presence of ammonia. After filtration, the solution was evaporated to small bulk under reduced pressure in a stream of hydrogen and finally brought to dryness in a vacuum-desiccator. The product obtained was a brown resin which was completely soluble in absolute methyl alcohol and gave all the melanogen reactions; yield: 0·91 g. It thus corresponded to the substance "A" from human melanotic urine. No substance equivalent to "B" was found.

The material was redissolved in 20 ml. distilled water, and 30 ml. mercuric acetate reagent were added. The precipitate was filtered off, washed, suspended in water, mixed with a little baryta paste and saturated with hydrogen sulphide. When absorption of the gas ceased, the liquid was filtered from mercuric sulphide. It was found that the presence of barium in the solution complicated the further treatment of the product, so ammonium carbonate was added to precipitate the barium and the liquid refiltered. The filtrate was evaporated to dryness in the usual way. The residue (corresponding to "D" from human melanotic urine) smelt strongly of ammonium acetate. This was driven off by re-dissolving in 250 ml. water and repeating the evaporation to dryness. The product was a hard, pale brown, amorphous solid; yield: 0·19 g. Its solution in methyl alcohol deposited fern-shaped crystals of ammonium chloride. To remove this impurity, 0·11 g. of the material was dissolved in water with an equal weight of potassium carbonate and the solution allowed to evaporate in a vacuum-desiccator. The dry residue was extracted with absolute methyl alcohol (in which potassium carbonate and chloride are insoluble) and the solution brought to dryness in a desiccator. The resulting potassium salts formed a pale brown, very hygroscopic, amorphous solid, soluble in absolute methyl alcohol, but not in ether, light petroleum or benzene; yield: 0·11 g. It gave intense Ehrlich, Thormählen, and ferric chloride reactions and also Ehrlich's diazo-reaction. The material was analysed quantitatively for sulphur. (Found: total sulphur, 2·48 %; sulphur in form of ethereal sulphate, 1·96 %.) It was apparently stable towards alkalis, but on warming with hydrochloric acid it blackened with formation of melanin.

Substances corresponding to the products "C" and "E" from human melanotic urine were also obtained; yields: 0·03 g. and 0·72 g. respectively. As these substances gave the melanogen reactions very weakly or not at all they were not examined any further.

III. *The administration of 5 : 6-dihydroxyindole-2-carboxylic acid.*

Having produced a typical melanotic urine by the injection of 5 : 6-dihydroxyindole into rabbits, it was of interest to see if the injection of a closely related compound, 5 : 6-dihydroxyindole-2-carboxylic acid, would lead to a similar result. This acid is a colourless material which readily oxidises to melanin on exposure to air, and Raper [1927] has shown that it arises in the tyrosinase-tyrosine reaction under certain conditions. A solution of 5 : 6-dihydroxyindole-2-carboxylic acid was prepared according to the method described by Raper and concentrated to about 10 ml. by evaporation under reduced pressure in a stream of carbon dioxide. After filtration from unchanged tyrosine, the liquid was injected subcutaneously into a rabbit. It was noted that the solution showed some marked differences in its behaviour from a solution of 5 : 6-dihydroxyindole. While the Ehrlich, ferric chloride, and hydrochloric acid reactions were given in the usual way, the Thormählen reaction was negative and on the addition of alkali the colour of the solution changed to brown but did not become black. The substance was precipitated by basic lead acetate and by Hopkins's reagent. Following the administration of the carboxylic acid the rabbit excreted a urine which was not a typical melanotic urine, though it resembled one in certain respects. The urine darkened on exposure to air and gave the Ehrlich, ferric chloride and hydrochloric acid reactions. On warming with caustic soda, however, the colour changed to brown—melanotic urine is stable to alkali—whilst the Thormählen reaction was negative. The chromogenic substance could be completely precipitated from the urine by basic lead acetate and by Hopkins's reagent. On decomposing the lead or mercury precipitate with hydrogen sulphide, the solution obtained gave the same reactions as the original urine. These properties strongly suggest that the 5 : 6-dihydroxyindole-2-carboxylic acid was excreted unchanged. In any case, it is obvious that the substance is not produced in human melanuria and is unlikely to be the precursor of the melanogen.

IV. *The separation process applied to normal rabbit urine.*

In order that an indication might be gained of the extent to which the chromogenic substances were separated from other constituents of urine by the precipitation process used in the foregoing experiments, normal rabbit urine was worked up by the same method. 8·29 g. basic lead acetate precipitate were prepared from 400 ml. urine and treated with carbon dioxide as previously described. Corresponding to the substance "A" a very hygroscopic pale brown amorphous solid was obtained, containing sulphur and nitrogen; yield: 1·53 g. The Thormählen, Ehrlich and ferric chloride reactions were negative, but the ferricyanide reaction was strongly positive. A solution of 1·38 g. of this material on precipitation with the mercuric acetate reagent gave products corresponding to "D" and "E"; yields: 0·10 g., 1·25 g., respectively. The former substance was a brown gummy residue completely soluble in water and absolute methyl alcohol, insoluble in ether and benzene, and containing nitrogen and sulphur.

The Ehrlich, Thormählen, ferric chloride and biuret reactions and tests for the presence of tryptophan, indoxyl, allantoin, and ethereal sulphates were all negative. Ehrlich's diazo-reaction succeeded, whilst on applying Tollens's test for glycuronate the substance gave a positive, though weak, reaction.

The product corresponding to "E" was a hygroscopic syrup containing nitrogen and sulphur and giving a weak ferric chloride reaction, Ehrlich's diazo-reaction and Tollens's test for glycuronates. It was not an ethereal sulphate. Only a minute amount of material corresponding to the substance "B" from human melanotic urine was isolated, and although 0·09 g. of a substance corresponding to "C" was obtained, it was a yellow resin which did not resemble in any way the product in the human case.

These experiments show that a substance is excreted in normal rabbit urine which cannot be separated from the melanogen by any of the processes applied by Eppinger and later workers, for in its solubility and precipitation reactions it behaves in the same way as the chromogenic material. Whether this substance is excreted in human urine has not yet been tested, but the possibility must be borne in mind.

V. *Preparation of the ethereal sulphate of 2 : 6-dihydroxyindole.*

This was carried out by a method similar to that used by Jolles and Schwenk [1915] for making indoxyl-potassium sulphate. 2 g. tyrosine in 4 l. water were oxidised by means of tyrosinase at p_H 6·5 as described previously [Raper, 1927]. When the maximum red colour had developed, 1 ml. of 10 % acetic acid was added and the solution filtered. The filtrate was evacuated in large filter flasks and well shaken to remove dissolved gases. It was then kept under hydrogen until the red colour had disappeared, this indicating the formation of 5 : 6-dihydroxyindole. After adding a few ml. of sulphurous acid and a little capryl alcohol dissolved in ethyl alcohol the solution was concentrated by distillation under reduced pressure in an atmosphere of hydrogen. During this concentration a second similar batch of material was prepared and this was evaporated in the same flask as the first. When the liquid had been reduced in volume to about 300 ml., unchanged tyrosine (1·37 g.) which had separated out was filtered off. The filtrate was concentrated further to about 20 ml. and again filtered. The filtrate was taken to dryness and the residue extracted with 30 ml. of warm pyridine. Only a small portion dissolved. This pyridine solution containing 5 : 6-dihydroxyindole was added slowly to a mixture of 30 ml. pyridine and 4 ml. chlorosulphonic acid containing 20 ml. of chloroform. It was allowed to stand at room temperature for 2 days. An equal volume of water was now added and the liquid distilled under reduced pressure to remove chloroform and pyridine. 11·5 g. caustic potash dissolved in a minimum volume of water were added and the potassium sulphate which separated was filtered off. The filtrate was at once saturated with carbon dioxide and the residual pyridine removed by distillation *in vacuo*. To the pigmented solution, which now measured 35 ml., 40 ml. absolute alcohol were added. This precipitated some potassium sulphate and carbonate as well as pigment, which were filtered off and washed with 50 % alcohol. To the filtrate an equal volume of alcohol was added which removed principally potassium sulphate. The precipitate was extracted with boiling 95 % alcohol and the extract added to the filtrate. The two were now concentrated by distillation *in vacuo* and finally in a vacuum-desiccator. A little KCl separated and was filtered off. The filtrate now measured 10 ml. It gave all the melanogen reactions of melanotic urine except that with $FeCl_3$ in the cold. It did, however, darken on heating with $FeCl_3$ and HCl. The filtrate was now precipitated with 100 ml.

alcohol and the white crystalline precipitate dried *in vacuo*. It contained the substance giving the melanogen reactions as well as potassium chloride and sulphate. After several trials it was found that the inorganic potassium salts could be separated by converting them all into the sulphate and precipitating this with 70 % alcohol in which the 5 : 6-dihydroxyindole potassium sulphate was moderately soluble. The precipitate containing the melanogen was dissolved in water and a slight excess of silver sulphate added. The solution was left overnight and the silver chloride filtered off. The filtrate was made faintly alkaline with potassium carbonate to remove the excess of silver, filtered through pulp and concentrated to 60 ml. by distillation *in vacuo*. Absolute alcohol was added to make the concentration 70 % and the solution left for some hours to allow K_2SO_4 to separate. This was filtered off and the filtrate concentrated by distillation *in vacuo* and then in a vacuum-desiccator. A small amount of amorphous material was filtered off and the solution was then returned to the desiccator and taken to dryness. The residue was dissolved in 2 ml. water and allowed to evaporate slowly in a desiccator over sulphuric acid at the ordinary pressure. A white crystalline substance separated which gave the Ehrlich and Thormählen reactions but still contained a little K_2CO_3. This was removed by redissolving in water, adding the mother-liquor and diluting to 20 ml. The solution was boiled and 5 % sulphuric acid added until it was exactly neutral to litmus. After cooling, 3 vols. of alcohol were added and after standing the K_2SO_4 was filtered off. The filtrate was taken to dryness *in vacuo*. The residue was dissolved in 1 ml. hot water, and 5 ml. hot alcohol were added. On cooling rosettes of needles separated out which were dried *in vacuo*. They weighed 63 mg. Analysis showed that they were the expected dipotassium sulphate of 5 : 6-dihydroxyindole. (Found by micro-analysis, N, 4·15; S, 16·00 %. $C_8H_5N(SO_4K)_2$ requires N, 3·6; S, 16·6 %.) A trace of the substance dissolved in a few ml. water gave strong Ehrlich and Thormählen reactions, but it did not darken with ferric chloride except when heated in strongly acid solution. The latter behaviour is unlike that of the melanogen of melanotic urine which rapidly darkens in the cold when ferric chloride alone is added.

An attempt was made to prepare the ethereal sulphate from the products of the enzyme action which had not dissolved in warm pyridine as described above. The residue insoluble in pyridine was heated in a water-bath under a reflux condenser with a mixture of 15 ml. pyridine, 75 ml. chloroform and 4 ml. chlorosulphonic acid for 10 hours. On working up the product in the same way as described for the pyridine-soluble material practically no substance giving the Ehrlich and Thormählen reactions was obtained.

DISCUSSION.

It is very probable that the melanogen produced by the administration of 5 : 6-dihydroxyindole to rabbits is identical with the chromogen of human melanuria. The artificially produced melanotic urine possessed all the characteristic properties of human melanotic urine, whilst the chromogenic material could be separated from both the human and rabbit urines by the same method and showed the same colour reactions, precipitation reactions and solubility in organic solvents in each case. Unfortunately, no conclusions could be drawn from an analysis of the products as they were certainly not pure. It has been demonstrated that a non-chromogenic material, exactly similar to the melanogen in its solubility in organic solvents and precipitation with salts of lead and mercury, occurs in normal rabbit urine and must therefore have been admixed with the rabbit melanogen; it may also have been present along with the human melanogen.

In their general properties the human and rabbit melanogens we have studied closely resemble the ether-insoluble chromogens described by Eppinger [1910], Feigl and Querner [1917], and Fürth and Kaunitz [1932]. The fact that some preparations of the chromogen obtained by Fürth and Kaunitz failed to give the Thormählen reaction was possibly due to a partial decomposition of the melanogen; in the present investigation preparations have been made which decomposed so far as to lose the Thormählen reaction without loss of the others. On the other hand, Eppinger's crystalline product failed to show the Ehrlich reaction while it still gave the Thormählen reaction. As we have already noted, however, there is some reason for doubting whether this substance was actually a melanogen. The existence of ether-soluble chromogens may also be questioned. Previous investigators have inferred their presence from the fact that methyl alcoholic solutions, from which the melanogen has been precipitated by ether, may retain the chromogen reactions. The melanogen is very soluble in methyl alcohol and it is reasonable to suppose that the addition of ether will not remove it completely from solution.

The foregoing experiments strongly suggest that the chromogen is a simple derivative of 5 : 6-dihydroxyindole. The latter substance shows many of the properties associated with the melanogen, including the ready oxidisability to melanin and the positive Ehrlich and Thormählen reactions—the latter reaction is not given by such closely related compounds as the 2-carboxylic acid and the *N*-methyl derivative. In the body it is converted into the chromogen; that this is not a general property of 5 : 6-dihydroxyindole derivatives is shown by the fact that the 2-carboxylic acid fails to produce a melanogen under such conditions. The stability of the chromogen towards alkali proves that it cannot be 5 : 6-dihydroxyindole itself. Now phenolic and similar hydroxylic compounds are excreted in the urine chiefly in combination with sulphuric or glycuronic acid. 2-Hydroxyindole, for example, is excreted as indican. It is therefore probable that if 5 : 6-dihydroxyindole were to arise in the body it would be excreted in the form of an ethereal sulphate or glycuronate. Such derivatives would be stable towards alkalis and readily hydrolysed by acids to 5 : 6-dihydroxyindole which would then oxidise to melanin. The behaviour of the melanogen would be explained by this hypothesis and there is a certain amount of evidence that it represents the actual state of affairs. In the present investigation a small percentage of ethereal sulphate was always associated with the chromogen and accounted for about 80 % of the total sulphur content. The dipotassium disulphate of 5 : 6-dihydroxyindole is, however, more stable than the chromogen of melanuria; it does not darken with ferric chloride unless heated in strongly acid solution. It is, of course, quite possible that the monosulphate is a more unstable compound. Glycuronates were always found with the melanogen, though it must be remembered that, in the experiments with rabbits at least, the melanogen could not be separated from a glycuronate occurring in normal urine.

The close relationship between the chromogen of melanuria and 5 : 6-dihydroxyindole is in agreement with the view that melanin is formed in the higher animals by the same series of changes that take place when tyrosine is oxidised by tyrosinase *in vitro*. Fürth and Kaunitz [1932] have pointed out that on this basis it is easy to account for the appearance of a catechol derivative in some melanotic urines by supposing that the decomposition of the 3 : 4-dihydroxyphenylalanine which is the first oxidation product of tyrosine can take place along two paths: either (i) there is oxidation to the quinone, followed by ring closure and the formation of 5 : 6-dihydroxyindole as previously described by Raper [1927], or else (ii) there is oxidation of the side chain in the usual way, leading to the formation of catecholacetic acid.

SUMMARY.

1. The chromogen of human melanuria has been studied and its separation from other constituents of urine by precipitation with lead acetate and mercuric acetate is described.

2. The administration of 5 : 6-dihydroxyindole to rabbits causes the excretion of a melanogen in the urine. The properties of this melanogen suggests its identity with the human melanogen.

3. 5 : 6-Dihydroxyindole-2-carboxylic acid does not produce a condition resembling human melanuria on administration to rabbits and is unlikely to be a precursor of the melanogen.

4. There is evidence that the chromogen is a simple derivative of 5 : 6-dihydroxyindole, possibly an ethereal sulphate or glycuronate.

5. The synthesis of 5 : 6-dihydroxyindole dipotassium disulphate is described.

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REFERENCES.

Bloch and Schaaf (1925). *Biochem. Z.* **162**, 181.
Bondzyński, Dombrowski and Panek (1905). *Z. physiol. chem.* **46**, 83.
—— and Panek (1902). *Ber. deutsch. chem. Ges.* **35**, 2959.
De Coulon (1920). *Compt. Rend. Soc. Biol.* **83**, 1451.
Eppinger (1910). *Biochem. Z.* **28**, 181.
Feigl and Querner (1917). *Deutsch. Arch. klin. Med.* **123**, 107.
Fürth and Kaunitz (1932). *Biochem. Z.* **253**, 231.
Gessard (1903). *Compt. Rend. Acad. Sci.* **136**, 1086.
Jolles and Schwenk (1915). *Biochem. Z.* **69**, 467.
Onslow (1915). *Proc. Roy. Soc. Lond.* B, **89**, 36.
Pugh (1933). *Biochem. J.* **27**, 475.
Raper (1927). *Biochem. J.* **21**, 89.
Thannhauser and Weiss (1922). *Kongr. inn. Med.* **34**, 156.

XI. BROMINE IN THE TISSUES.

By THEODORE FREDERIC DIXON[1].

From the Central Pathological Laboratory of the London County Mental Hospitals.

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THE findings of Zondek and Bier [1932, 1; 1933] with respect to the low blood bromine values of manic depressive psychotics have been criticised by many [Fleishhacker and Scheiderer, 1932; 1933; Hahn, 1933; Holtz and Roggenbau, 1933; and Dixon, 1934] on the ground that the method of bromine determination employed, that of Pincussen and Roman [1929], is untrustworthy. Since other investigators using the same chemical technique have confirmed these results on manic depressives [Urechia and Retezeanu, 1933; Sacristan and Peraita, 1933] or have found very constant values for normal blood [Kuranami, 1932] it seemed desirable to repeat these determinations, using the method of Dixon [1934]. It was thought also that confirmation was needed for the theory that a bromine-containing compound was stored in the pituitary which circulated in the blood causing sleep and sedative effects [Zondek and Bier, 1932, 2]. This theory followed on the earlier findings of Bernhardt and Ucko [1926] who claimed that the pituitary had a bromine content 15–30 times that found in blood.

It was therefore decided to investigate the variability of normal human blood bromine values. The results below were obtained with donors including medical officers, laboratory co-workers, *etc.*

D.	0·53 mg./100 ml.	McC.	0·986 mg./100 ml.
N.C.	0·42 ,,	C.N.	1·358 ,,
Hst.	0·63 ,,	C.	0·78 ,,
How.	0·71 ,,	P.	0·84 ,,
Mort.	0·69 ,,	I.	0·39 ,,

The values are so widely divergent that it might be thought, in view of the constancy of the blood bromine claimed by other workers, that errors had been made in the experimental technique or in the selection of the blood donors. Assuming that the experimental technique is correct, the possibility that the blood donors had been subjected to some form of bromide therapy must still be excluded. Since it is known that any ingested bromide is excreted from the body very slowly especially if the food is low in chloride [Hastings *et al.* 1932; Palmer and Clark, 1932] great care was exercised to ensure that the blood donors had received no bromide or iodide for over six months previous to the taking of the blood sample. This could be done easily in the normal series. In the case of inmates of mental hospitals where case-books were the only record there was necessarily less certainty. It was further found almost impossible to rely on blood samples from in-patients of a general hospital. Thus a group of 12 in-patients at King's College Hospital, untreated with bromide since admission, were investigated with respect to their blood bromine, using Walter's [1924] approximate clinical method for the determinations. In two cases the blood bromine was over 10 mg./100 ml. (The clinical method does not detect values of

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[1] Maudsley Research Fellow.

1 mg./100 ml.) These facts indicate some of the difficulties in the interpretation of values obtained for the bromine content of blood or tissues if the possibility of bromide dosage is not considered. They also make it probable that the variations in normal series are due to differences in bromine intake.

Little systematic work on the bromine content of common foodstuffs has been done, although von Damiens and Blaignan [1931; 1932], using methods for bromine estimation which are not grossly inaccurate, have demonstrated its presence in varying amounts throughout the vegetable kingdom. The abbreviated table below, taken from their results, shows a wide range of variation both in the absolute bromine amount and in its relation to the corresponding chloride content.

	mg. Br/100 g. dry weight	g. Cl/100 g. dry weight	Br/Cl
Bread	0·09–0·61	0·82–1·8	0·0007–0·000046
Potatoes	0·27–1·43	0·63–0·319	0·0004–0·004
Lentils	1·02	0·012	0·083
Melon	9·45–26·2	0·765–1·04	0·012–0·025

Cooking salt contains small amounts and the present author has found a sample of such salt to contain 1 mg. Br/g. Cl, or Br/Cl = 0·001. It is thus easy to understand why the normal human blood bromine varies from one individual to another when the bromine and chlorine intakes may be widely different.

A series of bromine determinations were next done on the blood of manic depressive psychotics, untreated with bromide or iodide for at least six months previously.

Lark.	1·716 mg./100 ml.	Lo.	0·453 mg./100 ml.
Est.	0·328 ,,	Sh.	0·78 ,,
Ed.	0·512 ,,	Si.	0·90 ,,
Pr.	0·572 ,,	M.	0·59 ,,
Ant.	1·64 ,,	McD.	0·46 ,,
Ps.	0·28 ,,	W.	0·97 ,,

It is clear that these individuals exhibit the same type of variability in their blood bromine as do normal persons.

Bromine determinations were then carried out on human and animal hypophyses to find out if these glands really contained high amounts of bromine (15–30 mg./100 g. wet weight) as stated by Zondek *et al.* The method previously described for blood was used except that the tissues were desiccated so that they could be stored before the analyses without fear of decomposition. Amounts of 1–2 g. dry tissue were used for the determinations as preliminary experiments on ox hypophyses and showed only a trace of bromine. One gram of dry human hypophysis represents 5–6 g. wet tissue equal to approximately 10 human hypophyses (average wet weight of each hypophysis 0·5 g.). A preliminary alkaline hydrolysis for 4 hours on the water-bath was employed before evaporation and ashing as in the blood determinations. The human hypophyses, large numbers of which were obtained from mental hospitals, were divided into groups according to type of mental disorder. No division into groups according to age or sex was made. Table I shows that considerable variation in the bromine contents of different batches of pituitaries occurs. Unless otherwise stated, the pituitaries were obtained from patients who had had no bromide during the last six months of life. The only ones which showed excessive amounts of bromine comparable with the values of Zondek were those from mental hospital patients who had definitely received bromide during life.

Table I. *Bromine determinations on batches of hypophyses.*

	mg. Br/100 g. dry weight	mg. Br/100 g. wet weight calculated
Ox	2·8	0·6
Pig	1·35	0·27
Human:		
(1) G.P.I.	8·27	1·65
(2) Idiots	2·10	0·42
(3) Miscellaneous insane	6·97	1·39
(4) Normals	4·22	0·84
(5) Normals	11·85	2·39
(6) Miscellaneous insane (known to have received bromide)	46·5	9·84
(7) " " "	133	26·6
(8) Seniles	3·53	0·7
(9) "	6·77	1·35
(10) Melancholics	8·46	1·69
(11) Manics	9·1	1·8
(12) Miscellaneous insane (some possibility of bromide dosage)	27·9	4·7
(13)	26·1	4·4

These results show then that the amount of bromine normally in the pituitary, which is of the same order as that in blood, is inconsistent with the belief that a bromine-containing substance is either stored or secreted by that organ.

It was then decided to investigate the relationship between bromine and chlorine in the animal body and as it was thought that *post mortem* human material was not reliable, pigs, which were available, were chosen. The organs of two pigs were examined with respect to their bromine and chlorine contents. The method of Dixon [1934] was employed for the bromine estimations, using 10–15 g. tissue, and the method of Eisenman [1929] for the chlorine estimations, using about 2 g. fresh tissue.

The results are shown in Table II.

Table II. *Results of bromine and chlorine determinations, in mg. per 100 g. tissue, on pig's organs.*

	Boar			Gilt		
Organ	Bromine	Chlorine	Br/Cl	Bromine	Chlorine	Br/Cl
Serum	1·25	314	0·004	0·75	320	0·0023
Kidney	0·445	203	0·0022	0·361	197	0·0018
Lung	0·551	218	0·0025	0·397	216	0·0018
Ovaries	—	—	—	0·647	—	—
Testes	0·334	184	0·0018	—	—	—
Liver	0·295	104	0·0028	0·213	101	0·002
Brain	0·192	126	0·0016	0·191	142	0·0014
Pancreas	0·259	110	0·0025	0·265	109	0·0025
Adrenal	0·368	107	0·0035.	—	—	—
Pituitary	0·270	145	0·0019	—	—	—

The pituitary figures, which represent duplicate determinations on groups of twenty pooled organs obtained from pigs in the same sties, are clearly not very different from those found for other organs. These two pigs had been fed for 5 months previous to death on a diet, the essential constituents of which, from the standpoint of bromine and chlorine content, are given below. A rough estimate of the relative bromine and chlorine content of this diet was made, using the results of von Damiens and Blaignan [1931] and those of the present author on cooking salt.

Constituent	Barley meal	Wheat middlings	Salt	Total
% in diet	60	35	0·33	
Cl g./100 g.	0·050*	0·06*	60·7†	
Cl g./100 g. diet	0·03	0·021	0·20	0·251 (g.)
Br mg./100 g.	0·45*	0·18*	60·7†	
Br mg./100 g. diet	0·27	0·063	0·20	0·533 (mg.)

* Data of von Damiens and Blaignan [1931]. † Data of the author.

From this table it will be seen that the Br/Cl ratio $(0{\cdot}533/0{\cdot}251 \times 1000 = 0{\cdot}002)$, although only very approximate, is roughly the same as that found in the pig tissues.

CONCLUSIONS.

Determination of the bromine distribution in the animal body leaves its rôle in the organism as mysterious as ever. It seems probable that its presence is fortuitous and dependent on food intake. No evidence has been obtained in support of the theory that a bromine-containing hormone exists in the pituitary. Bromine metabolism does not appear to bear any relation to mental disease.

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REFERENCES.

Bernhardt and Ucko (1926). *Biochem. Z.* **170**, 461.
von Damiens and Blaignan (1931). *Compt. Rend. Acad. Sci.* **193**, 1460.
—— —— (1932). *Compt. Rend. Acad. Sci.* **194**, 2077.
Dixon (1934). *Biochem. J.* **28**, 48.
Eisenman (1929). *J. Biol. Chem.* **82**, 411.
Fleishhacker and Scheiderer (1932). *Klin. Woch.* **11**, 1550.
—— —— (1933). *Monatschr. Psychiat.* **84**, 348.
Hahn (1933). *Klin. Woch.* **12**, 390.
Hastings, Harkins and Liu (1932). *J. Biol. Chem.* **94**, 681.
Holtz and Roggenbau (1933). *Klin. Woch.* **12**, 1410.
Kuranami (1932). *J. Biochem.* **15**, 205.
Palmer and Clark (1932). *J. Biol. Chem.* **99**, 435.
Pincussen and Roman (1929). *Biochem. Z.* **207**, 416.
Sacristan and Peraita (1933). *Arch. Neurobiol.* **1**, 39.
Urechia and Retezeanu (1933). *Compt. Rend. Soc. Biol.* **112**, 411.
Walter (1924). *Biochem. Z.* **149**, 145.
Zondek and Bier (1932, 1). *Klin. Woch.* **11**, 633.
—— —— (1932, 2). *Klin. Woch.* **11**, 759.
—— —— (1933). *Klin. Woch.* **12**, 55.

XII. THE BODY FATS OF THE PIG.

IV. PROGRESSIVE HYDROGENATION AS AN AID IN THE STUDY OF GLYCERIDE STRUCTURE.

By THOMAS PERCY HILDITCH and WILLIAM JOHN STAINSBY.

From the Department of Industrial Chemistry, University of Liverpool.

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It was pointed out in an earlier communication in this series [Banks and Hilditch, 1932] that the relationship between the content of fully-saturated glycerides and the degree of saturation as a whole of pig, ox and sheep depot fats and also cow milk fats (a group in which the saturated fatty acids ranged from 34 to 72·5 mols. % of the total fatty acids in each fat) was closely similar to that of a series of progressively hydrogenated cottonseed oils. Cottonseed oil resembles the most unsaturated type of pig body fat in so far as it contains about 25 % of saturated (almost all palmitic) and 75 % of unsaturated acids, all the saturated acids being present in the form of mixed, saturated-unsaturated glycerides. In the present paper the glyceride structure of a similar group of hydrogenated pig fats is described, since it was considered of some interest to compare the increase in fully-saturated glycerides, in fats produced by progressive hydrogenation of an animal depot fat of the most unsaturated type, with that observed in the series of natural lards, tallows and butters.

The experimental material required for this purpose was also suitable for the study of other features of the glyceride structure of the original pig fat employed. We have been able to estimate, by two independent methods, the total content therein of glycerides consisting wholly of C_{18} (stearic, oleic and/or linoleic) acids, to show that the monopalmitoglycerides of the fat were almost entirely, if not wholly, β-palmito-$\alpha\alpha'$-diacylglycerides, and finally, to give a more detailed statement than previously of the probable proportions of the chief component glycerides of the original fat.

The inner layers of the pig back fat, the component acids of which had already been examined [Dean and Hilditch, 1933] and found to be practically the same throughout the layer, served as the primary material for the present study. Owing to the large quantity of material required by the present work, it was necessary to commence from the inner back fat; the amount of the outermost layer of back fat (which would have provided a still better starting-point, owing to its somewhat greater unsaturation) was not sufficient to provide the requisite quantities of all the desired hydrogenated products.

The mixed inner back fat had a mean saponification equivalent of 286·2 and a mean iodine value of 63·0. Its mixed fatty acids (setting-point 40·7°) were analysed by the ester-fractionation method; the results, together with the corresponding figures of Dean and Hilditch [1933] for the outermost (I), middle (II) and innermost (III) layers of the inner back fat, are given in Table I.

Portions of the fat were hydrogenated to varying degrees by agitation with a small proportion of nickel on kieselguhr in hydrogen at atmospheric pressure at 175–180°, and products of the following iodine values were thus prepared: 48·9, 43·0, 35·8 (800 g. of each), and 28·9, 18·7, 10·0 and 0·2 (400 g. of each).

Table I. *Component acids of original inner back fat.*

Acid	Weight percentages				Molar percentages			
	I	II	III	Mixed	I	II	III	Mixed
Myristic	2·9	2·8	3·0	2·3	3·5	3·3	3·6	2·8
Palmitic	24·9	25·5	24·6	25·6	26·6	27·3	26·2	27·3
Stearic	14·5	14·5	14·5	14·9	14·0	14·0	14·0	14·4
Oleic	42·7	41·3	42·8	42·2	41·4	40·1	41·6	40·9
Linoleic*	13·9	14·5	13·7	13·8	13·6	14·2	13·4	13·5
C_{20-22} unsaturated	1·1	1·4	1·4	1·2	0·9	1·2	1·2	1·1

* Some of the C_{18} unsaturated esters isolated from the original pig fat were examined qualitatively. It was found that the oleic and linoleic acids present were respectively the common forms of $\Delta^{9:10}$-octadecenoic and $\Delta^{9:10,12:13}$-octadecadienoic acids. Oxidation with dilute alkaline permanganate gave 9:10-dihydroxystearic acid, M.P. 131°, and two tetrahydroxystearic acids, M.P. 154° and 172°; a tetrabromostearic acid which was sparingly soluble in light petroleum and melted at 114° was also obtained. The yields of tetrabromo- and tetrahydroxy-stearic acids were of the same order as those given by the linoleic acid from a seed-fat such as cottonseed oil.

Sufficient of each fat (including the original) was oxidised with potassium permanganate in acetone to yield a suitable quantity of fully-saturated glycerides to permit of the component acids of the latter being analysed by the ester-fractionation method with the results[1] shown in Table III.

In Table III are also given the fatty acid compositions of each of the hydrogenated fats. To facilitate comparison between the various products, the latter have been calculated from the original analysis (Table I) in conjunction with the observed iodine values of the fats. This course has been adopted in order to avoid the slight variations otherwise introduced by the small but inevitable experimental errors of analysis [*cf.* Hilditch, 1934]. In view of the good agreement between the four analyses recorded in Table I the figures for the original fat must be considered to be a close approximation to the truth.

We have however made independent ester-fractionation analyses of the component fatty acids of the fats of iodine value 48·9, 35·8, and 10·0 (Table II). The direct analysis of the first-named fat was essential in order to ascertain the extent to which linoleic glycerides were undergoing hydrogenation. The result showed that the hydrogenation had followed the usual, mainly selective course and indicated that linoleic acid would no longer be present in the remaining, more saturated products.

Table II. *Component fatty acids of hydrogenated pig fats (by direct analysis) (mol.* %*).*

	48·9	35·8	10·0
Iodine value of fat	%	%	%
Myristic	2·8	2·2	1·3
Palmitic	25·6	28·1	29·0
Stearic	18·1	28·2	56·3
Oleic (and *iso*-oleic)	50·9	40·5	11·9
Linoleic	0·9	—	—
C_{20-22}	1·7	1·0	1·5

The complete analytical data for each hydrogenated product and its corresponding fully-saturated components may now be considered. In Table III, in which these are summarised, are included the weights of fat from which the fully-saturated glycerides were obtained, and the weights of the esters of the mixed acids of the latter which were subsequently fractionated.

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[1] All percentages in Table II *et seq.* are molar, not weight.

Table III. *Fully-saturated glycerides and component fatty acids of the hydrogenated pig fats.*

Iodine value of fat	63·0*	48·9	43·0	35·8	28·7	18·7	10·0
Component fatty acids (mol. %) in whole fat:†							
Myristic	2·8	2·8	2·9	2·8	2·8	2·8	2·8
Palmitic	27·3	27·4	27·4	27·4	27·5	27·5	27·5
Stearic	14·4	15·7	21·4	29·6	36·2	47·5	57·4
Oleic (and *iso*-oleic)	40·9	52·1	47·3	39·2	32·5	21·2	11·3
Linoleic	13·5	1·0	—	—	—	—	—
C_{20-22}	1·1	1·0	1·0	1·0	1·0	1·0	1·0
Fully-saturated glycerides.							
Weight of whole fat oxidised (g.)	401·8	404·8	308·2	302·7	103·0	100·5	100·0
Fully-saturated glycerides (mol. %)	5·6	7·1	10·9	19·6	32·0	53·2	71·1
Component acids (mol. %) in fully saturated glycerides:							
Myristic	—	1·3	0·5	—	—	—	—
Palmitic	54·0	47·3	44·7	41·2	40·4	38·9	36·7
Stearic	46·0	51·4	54·8	58·8	59·6	61·1	62·2
C_{20-22}	—	—	—	—	—	—	1·1
(Weight of fully-saturated esters fractionated (g.)	18·8	23·0	25·1	27·5	27·0	23·0	27·0)

* Original fat.

† Calculated from iodine value of fat and analysis of acids from original fat of iodine value 63·0.

Comparison of the proportions of fully-saturated components in the partially-hydrogenated pig fats and in natural depot (and milk) fats of similar relative unsaturation.

The relation between the molar content of fully-saturated glycerides and the molar percentages of saturated acids in the total fatty acids of the various fats is shown graphically in Fig. 1. The points indicated by letters refer to the different natural fats (depot fats of pig (L), ox and sheep (T), or butter fats (B)), whilst the unbroken curve has been drawn through the values obtained from the hydrogenated pig fats (Table III). The dotted curve represents the relationship which would obtain if the amount of fully-saturated components in a fat were proportional to the cube of the proportion of saturated acids present in the mixed acids. The broken line shows the fundamentally different relations subsisting in seed-fats, in which fully-saturated glycerides are rarely found in greater amount than that necessary to leave a ratio of about 55–60 % of saturated to 45–40 % of unsaturated acids linked together as mixed saturated-unsaturated glycerides [Collin and Hilditch, 1929].

In fats hydrogenated by metallic catalysts under the conditions which we have used, the linoleic glycerides are very largely converted into oleic derivatives before the stearic content is appreciably augmented. Thus the molar percentage of linoleic acid in the component fatty acids fell from 13·5 to 1, while the increase in stearic acid was less than 2 %, and the fully-saturated glyceride content only rose from 5·6 % to 7·1 %. In this respect the artificially hydrogenated fats form a different series from the natural depot fats in which stearic acid augments mainly at the expense of oleic acid, rather than of linoleic acid. This difference does not however affect the relationships now under consideration, since these are based on the relative proportions of saturated to unsaturated acids as a whole in each fat.

It will be seen that, as in the case of hydrogenated cottonseed oils, the points given on the graph by the natural tallows and lards[1] follow the hydrogenated pig fat curve with a fair degree of consistency. The most arresting feature of the diagram is that, as with the hydrogenated fats, the series of values for the natural fats definitely tends to meet the horizontal axis at a point representing 25–30 % of saturated acids in the total fatty acids. This amount is also of course that of the approximately constant palmitic acid content which has been shown to be characteristic for animal depot fats and also butter fats. If the occurrence of fully-saturated glycerides in these fats were determined by the relative concentration of any one acid, or class of acid, the presence of 25–30 % of palmitic acid alone should suffice to produce 5 % or somewhat more of tripalmitin. Actually, tripalmitin has never been observed in either lards or tallows, the fully-saturated components being almost entirely mixed palmitostearins.

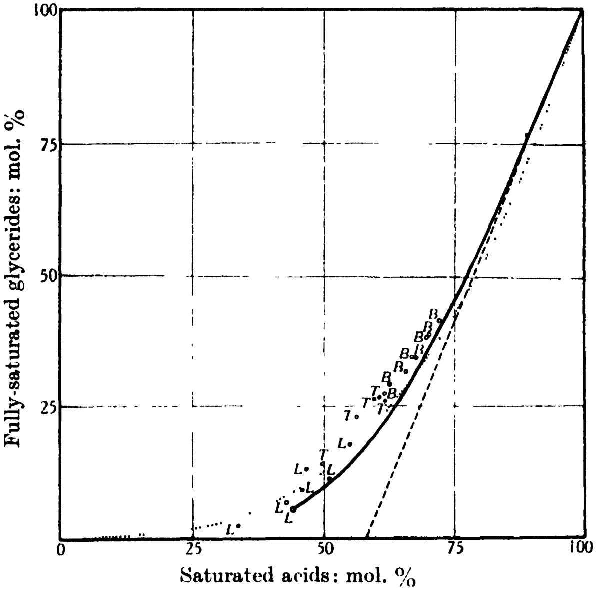

Fig. 1. *L*, pig fats. *T*, tallows. *B*, butter fats. —— Hydrogenated pig fat. - - - - - - Seed fats. · · · · $y \propto x^3$.

These facts, as well as the parallelism in general glyceride structure between the natural fats and the hydrogenated pig fats, accord with the suggestion made in an earlier paper [Banks and Hilditch, 1932] that, in the lard and tallow types of depot fats (with relatively high stearic contents), a mixture of triglycerides, formed initially from about 30 % of palmitic and about 70 % of oleic acids and containing little or no fully-saturated compounds, may undergo subsequent hydrogenation of some of the oleo-glycerides. Mere similarity of chemical structure in two series of compounds is of course no proof of the mechanism by which one of the series may have been produced; nevertheless, specific features in the structure of natural products merit due consideration when hypotheses as to their biosynthesis are elaborated.

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[1] Fig. 1 shows that the milk fats fall into line with tallows and lards from the point of view of their general glyceride structure, and it has also been found [Hilditch and Sleightholme, 1931, p. 518] that in these fats the united molar contents of the characteristic lower saturated fatty (butyric-lauric) acids together with stearic and oleic acids tend towards a constant figure of about 65 % (mol.)—little different from that of the total C_{18} acids in tallows and lards. There are therefore fundamental and quantitative similarities in the general constitutions of these milk and depot fats.

Content of tri-C_{18}-glycerides in the original pig fat.

Direct methods of determination of the triolein or triunsaturated glyceride content of a fat are still lacking, but it has recently been shown [Hilditch and Jones, 1934] that an approximate estimate of the total tri-C_{18}-glycerides present can be arrived at in suitable cases from the mean equivalents of the fractions obtained by systematic crystallisation of the fat after complete hydrogenation. An independent method, which serves as a check on the foregoing process and which has not been previously described, depends on the fact that palmitostearo-oleins are hydrogenated preferentially to distearo-oleins [Hilditch and Jones, 1932] and that, consequently, after partial hydrogenation to a point at which no dipalmito-unsaturated glycerides are left, the non-fully-saturated glycerides are composed solely of monopalmitodistearins and tri-C_{18}-glycerides. Both these methods have been applied in the present investigation.

(i) *Fractional crystallisation of the completely hydrogenated pig fat* (*iodine value 0·2*). The completely hydrogenated fat (102 g.) was crystallised first from 10 volumes of ether and the separated solids were then crystallised from 15 volumes of ether, the latter process being repeated three times. The fractions obtained and their analytical characteristics are summarised in Table IV.

Table IV. *Fractional crystallisation of hydrogenated pig fat* (*102 g., iodine value 0·2*).

		Glycerides			Corresponding acids (freed from unsap.)	
No.	g.	M.P.	S.E.	I.V.	S.E.	I.V.
A	69·1	68°	289·2	—	—	—
B	5·5	68°	287·3	—	—	—
C	8·5	—	286·1	—	—	—
D	2·9	—	285·9	—	—	—
E	7·9	—	284·5	0·7	272·0	0·5
F	8·1	—	283·8	2·5	270·0	1·3

Assuming that fraction A is a mixture of tristearin (S.E. 296·7) and palmitodistearin (S.E. 287·3), and that the iodine values of the glycerides in fractions E and F are due to oleodistearin, it follows that the fat (102 g.) contained 14·3 g. tristearin and 0·5 g. oleodistearin, corresponding to a molar content of 14·1 % tri-C_{18}-glycerides in the whole fat[1].

(ii) *Estimation of tri-C_{18}-glycerides from some of the partially hydrogenated fats.* Before applying this method, it is necessary to ascertain, (*a*) whether any tristearin has already been formed and (*b*) at what point the absence of dipalmitoglycerides from the non-fully-saturated glycerides can be presumed. The production of tristearin can be followed by subjecting the fully-saturated glycerides of the fats to systematic crystallisation. Thus, the fully-saturated glycerides in the fat of iodine value 18·7 (*v. infra*) showed no tristearin content.

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[1] This method of examination is unfortunately not sufficiently delicate to take account of the minor component acids (myristic 2·8 %, C_{20-22} 1·1 %) present in the fat. Calculated from the saponification equivalents as combinations of palmitic and C_{18}-glycerides, the molar composition of the hydrogenated fat amounted to: tristearin 13·6 %, palmitodistearin 78·4 %, dipalmitostearin 7·5 % and oleodistearin 0·5 %. This corresponds with a total molar fatty acid composition of palmitic 31 % and C_{18}-acids 69 % (calculated from original ester-fractionation analysis of the original fat: non-C_{18}-acids 31·2 %, C_{18}-acids 68·8 %).

The second question can be answered approximately by examining the relative amounts of stearic and palmitic acids added at each stage of the hydrogenation to the fully-saturated components. This is illustrated in Table V, the (molar) data in which have been obtained from the detailed analysis made of the fully-saturated components of each of the hydrogenated fats.

Table V. *Increase in non-C_{18} and stearic acids in fully-saturated glycerides at successive stages of hydrogenation.*

Stages (Fall in I.V.)	Increase in total saturated acids	Increase in fully-saturated glycerides	Mols. (per 100 g. fat) added of		Ratio C_{18}/C_{16}
			C_{16}*	C_{18}	
63·0–43·0	7·2	5·3	1·95	3·35	1·7
43·0–35·8	8·1	8·7	3·15	5·55	1·8
35·8–28·7	7·7	12·4	4·8	7·6	1·6
28·7–18·7	11·3	21·2	7·8	13·4	1·7
18·7–10·0	9·9	17·9	6·2	11·7	1·9
10·0–nil	11·3	28·9	4·9	24·0	4·9

* Minor amounts of C_{14} or C_{20-22} acids included with C_{16}.

From Table V it appears that the remaining non-fully-saturated components of the fats of iodine values 18·7 and 10·0 both consist solely of monopalmito-di-C_{18}-glycerides and tri-C_{18}-glycerides, since the ratio of C_{18} : C_{16}-glycerides added is almost exactly 2 : 1. It is also seen from Table V that, during the further hydrogenation to iodine value 10·0, there was no indication of the formation of tristearin.

Considering the fats of iodine value 18·7 and 10·0, detailed analyses reveal the data quoted in Table VI.

Table VI. *Component acids (mol. %) in the fully-saturated and non-fully-saturated part of fats of iodine value 18·7 and 10·0.*

	Fat of I.V. 18·7			Fat of I.V. 10·0		
	Whole fat	Fully-saturated	Mixed saturated-unsaturated (by diff.)	Whole fat	Fully-saturated	Mixed saturated-unsaturated (by diff.)
Mols:	100	53·2	46·8	100	71·1	28·9
Myristic	2·8	—	2·8	2·8	—	2·8
Palmitic	27·5	20·7	6·8	27·5	26·1	1·4
Stearic	47·5	32·5	15·0	57·4	44·2	13·2
C_{20-22}	1·0	—	1·0	1·0	0·8	0·2
Oleic and *iso*-oleic	21·2	—	21·2	11·3	—	11·3

In the non-fully-saturated portion of the fat of iodine value 18·7, therefore, there are, by difference, 10·6 mols. of non-C_{18} acids (corresponding to 31·8 mols. of "palmito-di-C_{18}-glycerides") and 36·2 mols. of C_{18} acids. Since 21·2 mols. of C_{18} acids are required in the formation of the 31·8 mols. of mixed glycerides, there remain 15·0 % (mol.) in the form of tri-C_{18}-glycerides.

A similar calculation from the results for the other fat gives 15·7 % (mol.) tri C_{18}-glycerides.

The results of the estimations by methods (i) and (ii) are reasonably concordant and the proportion of tri-C_{18}-glycerides other than tristearin (triolein, oleodistearins, oleolinoleins or similar combinations) in the original fat is clearly about 15 % (mol.).

The occurrence of β-palmito-glycerides in the pig depot fat. Some evidence as to the configuration of the mono-palmitoglycerides of the original pig fat has been obtained from study of the fully-saturated components isolated from the fats which had been hydrogenated respectively to iodine values of 35·8 and 18·7. It has already been shown that, at these stages, no tristearin had been produced in the course of the hydrogenation. Systematic crystallisation from ether of the fully-saturated glycerides from each fat gave considerable proportions of a palmitodistearin which melted at 67–67·5° (Table VII).

Table VII. *Palmitodistearins isolated from the fully-saturated glycerides of hydrogenated pig fats.*

Hydrogenated fat	Fully-saturated glycerides crystallised	Palmitodistearin isolated				Composition of F.S.G. calculated from component acid analysis (Table III)	
						Palmitodistearin	Dipalmitostearin
I.V.	g.	g.	%	M.P.	S.P.	%	%
35·8	19·2	11·2	58·3	67·5°	287·4	77	23
18·7	22·9	15·8	69·0	67°	286·7	84	16

The melting-points of α-palmito-βα′-distearin and of β-palmito-αα′-distearin are respectively 63·5° and 68° [Bömer *et al.*, 1907; 1909; 1913; Amberger and Wiesehahn, 1923; Robinson *et al.*, 1932], and thus the data in Table VII show that the least soluble fractions isolated were β-palmito-αα′-distearin. Moreover the quantity of β-palmitostearin amounted to 76 % and 82 % of the whole of the palmitodistearin present in the respective fully-saturated glycerides and, in the case of the fat of iodine value 18·7, the palmitostearin actually isolated corresponds to about 60 % of the whole of the monopalmito-di-C_{18}-glycerides present in the original fat, although no particular effort was made to obtain quantitative recovery of all the monopalmitodistearin. In view of the relative ease with which the compounds of melting-point 67·5° were obtained and of the well-known difficulties attending the separation of mixtures of the isomeric palmitodistearins by crystallisation, these results are considered to point definitely to the presence, in the original pig fat, of β-monopalmito-glycerides, unaccompanied by any appreciable quantities of the α-monopalmito-isomerides[1].

The exclusive occurrence of one configurational form of a mixed glyceride, as suggested for pig depot fat by these results, may be less uncommon in natural fats than would perhaps be supposed. At all events, the present observations are paralleled by the similar presence of β-oleodistearin in a group of vegetable seed fats, including those of kokum butter and *Allanblackia Stuhlmannii* [Heise, 1897; Hilditch and Saletore, 1933], cacao butter [Fritzweiler, 1902] and Borneo tallow [Klimont, 1904].

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[1] Dr Banks has suggested to us that the preferential hydrogenation of palmitostearo-oleins to that of distearo-oleins may be due to relative ease of hydrogenation of oleic radicals attached to the α-glyceryl carbon atoms as compared with those attached to a β-glyceryl carbon atom. This possibility is at present being investigated in our laboratory.

The probable proportions of the chief components of the original pig depot glycerides.

The data which have now been discussed allow a somewhat fuller statement to be given than formerly as to the main components of a pig depot fat, subject to the following limitations:

(*a*) The methods employed are not sufficiently delicate to indicate the mode of union of the minor component acids (myristic and the C_{20-22} group) which form only 2 or 3 % of the total fatty acids. They serve however to define the chief combinations of the acids containing 18 carbon atoms with those containing other than 18 carbon atoms in the molecule; in the case of pig depot fat the latter group is very largely composed of palmitic acid, and it thus becomes possible to outline the chief combinations of palmitic and C_{18} acids which occur in the mixed glycerides.

(*b*) Since the methods employed involve hydrogenation, *i.e.* removal of the unsaturated group, it is impossible to discriminate, in what follows, between the linoleic and oleic radicals originally present in the fat. (For the sake of brevity the unsaturated glycerides are referred to in the paragraphs immediately following as “oleins,” “oleic” derivatives *etc.*, the use of inverted commas denoting that both oleic and linoleic, and not only the monoethenoid, compounds may be concerned.)

The data now available include:

(*a*) The proportion (*ca.* 5 %) of fully-saturated glycerides in the original fat, these being mixtures of dipalmitostearin and palmitodistearin.

(*b*) The proportion (*ca.* 15 %) of tri-C_{18}-glycerides present. These might be tri-“oleins,” stearodi-“oleins,” or distearo-“oleins”; the latter are least likely to be present, the monostearo-glycerides being probably the chief representatives of this group, but there may well be at least some triolein or mixed linoleo-oleins in the original fat (the fatty acids of which included 41 % oleic and 13·5 % linoleic acids). Estimates are given below for this group reckoned alternatively as all monostearodi-“oleins,” or as all tri-“oleins.”

(*c*) The remainder of the stearo-glycerides (corresponding to the balance of the total stearic acid unaccounted for in the form of tri-C_{18}-glycerides) must be either palmitostearo-“olein” or palmitostearins (tristearin being absent and the presence of distearo-“oleins” in significant quantities being ruled out as unlikely). The quantity of stearic acid combined as palmitostearins being known (*a*), the residue must be allotted to palmitostearo-“olein.”

(*d*) The monopalmito-derivatives have been shown to be β-palmito-glycerides.

Alternative possible compositions of the main glycerides of the fat can therefore be given, to a rough approximation, according to whether the tri-C_{18}-glycerides contain one stearic residue or not (Table VIII).

Table VIII. *The general composition of the glycerides of pig back fat (iodine value 63·0).*

	Tri-C_{18}-glycerides estimated as		
	(*a*) Stearodi-“oleins” % (mol.)		(*b*) Tri-“oleins” % (mol.)
(β-) Palmitodistearin	2		2
Dipalmitostearin	3		3
Tri-“oleins”	—	to	15
Stearodi-“oleins”	15	,,	—
(β-) Palmitostearo-“oleins”	21	,,	36
(β-) Palmitodi-“oleins”	57	,,	42
Dipalmito-“olein”	2		2

The most abundant constituents of this particular fat are the palmitodi-"oleins" (42–57 %), followed by palmitostearo-"oleins" (21–36 %) and tri-C_{18}-glycerides (15 %, mainly unsaturated).

The possible limits within which the three classes of monosaturated-di-unsaturated, disaturated-mono-unsaturated, and tri-unsaturated glycerides may be present in the fat have been considerably narrowed by the present procedure, in comparison with those which it was formerly possible to indicate by consideration only of the amount of fully-saturated glycerides present. This is shown in Table IX, which also includes the similar values indicated by the old and the present methods for the content of tri-C_{18}-glycerides other than tristearin.

Table IX. *Limiting values for certain classes of glycerides in the pig back fat.*

	(a) From F.S.G. content alone	(b) Present analysis
Mono-unsaturated-disaturated	23–59	23–38
Di-unsaturated-monosaturated	72–0	72–42
Tri-unsaturated	0–36	0–15
Tri-C_{18}-glycerides	10–52	15

Amberger and Wiesehahn [1923], who investigated lards of iodine values 51·5 and 54·7 by fractional crystallisation of the original and of the completely hydrogenated fats, suggested that they contained dipalmitostearin (2 %), palmitodistearin (3 %), oleodistearin (2 %), oleopalmitostearin (11 %) and palmitodiolein (82 %), with possibly some triolein. These figures differ from those given above chiefly in the low proportion assigned to palmitostearo-oleins; this is perhaps to be expected in consequence of the undoubted difficulty of obtaining anything approaching a quantitative separation of these glycerides from the more abundant palmitodi-"oleins" which accompany them.

The data of Table VIII refer to a pig back fat of the more unsaturated type. A more saturated fat, such as a perinephric fat with iodine value about 55, of course must be somewhat differently constituted. Such a fat contains about 14 % of fully-saturated components and its mixed acids include about 70 % C_{18}-acids (of which about 17 % may be stearic acid). The determination of its approximate composition, which would involve the execution of another series of analyses on the lines of those described in this paper, has not been undertaken. It may be pointed out however that (if the leaf fat should contain about the same proportion of tri-C_{18}-glycerides as that in the back fat which we have examined) palmitodi-"oleins" would still be the most prominent components (35–50 %), palmitostearo-"oleins" would probably be present in proportions not differing very greatly from those given in Table IX, whilst, of course, the amount of palmitodistearin would be augmented in consequence of the increase in fully-saturated glycerides as compared with the back fat (possibly distearo-"oleins" might be present in minor quantities).

It will be perceived that the quantitative description of the component glycerides of pig depot fat is still incomplete; nevertheless the work reported above permits us to deduce with some confidence the general relative proportions of the different classes of mixed glycerides (enumerated in Table VIII) for a single specimen of the depot fat, and to indicate the probable alteration in their respective proportions in other pig depot fats of a more saturated character.

SUMMARY.

Investigation of the composition of a pig back fat after its catalytic hydrogenation to varying stages of saturation has led to the following conclusions:

1. The increase in the fully-saturated glycerides in a series of animal fats (pig, ox, sheep depot fats and cow milk fats), in which the total content of saturated acids progressively increases, is very similar to that displayed by the artificially hydrogenated pig back fats. The values for the natural fats, when plotted graphically, suggest that fully-saturated components would be absent from a fat of this group which contained only 25–30 % of saturated acids (*i.e.* palmitic but no stearic acid).
2. Estimations by two different methods showed that the fat contained 15 % (mol.) of tri-C_{18}-glycerides (other than tristearin, which was absent). The precise nature of this group (triolein, oleolinoleins, stearodioleins, or oleodistearin) was not ascertainable, but it is probable that it consists mainly of stearodioleins with perhaps a small proportion of tri-unsaturated glycerides.
3. The monopalmito-compounds present in the fat consist very largely, and quite probably solely, of β-palmito-glycerides (β-palmitodi-"olein" and β-palmitostearo-"olein," with a small amount of β-palmitodistearin).
4. Palmitodi-unsaturated glycerides (chiefly palmitodiolein) are the chief components of the original fat investigated, of which they may form from 42 to 57 %; palmitostearo-"oleins" (21–36 %) and the tri-C_{18}-unsaturated (or semi-unsaturated) glycerides (15 %) are the next most abundant constituents.

The perinephric fats of the pig, which are more saturated, probably contain somewhat less of the palmitodi-"oleins" and not very different amounts of palmitostearo-"oleins," compared with the back fat; but there is a corresponding increase in the palmitostearins present.

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REFERENCES.

Amberger and Wieschahn (1923). *Z. Unters. Nahr. Genussm.* **46**, 276.
Banks and Hilditch (1932). *Biochem. J.* **26**, 298.
Bömer (1913). *Z. Unters. Nähr. Genussm.* **25**, 321.
—— and Heimsoth (1909). *Z. Unters. Nähr. Genussm.* **17**, 353.
—— Schlemm and Heimsoth (1907). *Z. Unters. Nähr. Genussm.* **14**, 90.
Collin and Hilditch (1929). *Biochem. J.* **23**, 1273.
Dean and Hilditch (1933). *Biochem. J.* **27**, 1950.
Fritzweiler (1902). *Z. Unters. Nähr. Genussm.* **5**, 1164.
Heise (1897). *Tropenpflanzer*, **1**, 10.
Hilditch (1934). *Biochem. J.* **28**, 779.
—— and Jones (1932). *J. Chem. Soc.* 805.
—— —— (1934). *J. Soc. Chem. Ind.* **53**, 13 T.
—— and Saletore (1933). *J. Soc. Chem. Ind.* **52**, 101 T.
—— and Sleightholme (1931). *Biochem. J.* **25**, 507.
Klimont (1904). *Monatsh. Chem.* **25**, 931.
Robinson, Roche and King (1932). *J. Amer. Chem. Soc.* **54**, 705.

XIII. THE ASCORBIC ACID CONTENT OF THE INTESTINE OF THE GUINEA-PIG.

By SYLVESTER SOLOMON ZILVA[1].

From the Division of Nutrition, Lister Institute, London.

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It is now established beyond doubt that certain organs and glands of animals whether susceptible to scurvy or not contain ascorbic acid in higher concentration than most of the other tissues. Thus the anterior lobe of the pituitary, the adrenals and to a lesser extent the liver have been shown by the reducing and by the biological methods to be rich in the vitamin. Harde and Wolff [1934], working with the mouse, and Hopkins [1934], working with the rat, indicated that the small intestine belongs to this group of organs. Giroud and Leblond [1934] found that silver nitrate when injected intravascularly is reduced by the intestine much more markedly in guinea-pigs on a mixed diet than in similar animals on a scorbutic diet, from which they deduce that the intestine of the former animals is also rich in ascorbic acid. The results to be produced confirm this conclusion.

When guinea-pigs are maintained on a scorbutic diet for some days, extracts of their tissues lose the capacity for reducing indophenol rapidly in acid solution. On the other hand, when ascorbic acid is offered *per os* or injected into the blood stream of such animals they acquire again this reducing property [De Caro, 1934]. The implication therefore is that the substance which reduces indophenol in this case at a more rapid rate than the other reducing substances present in the tissues is ascorbic acid.

In Table I are given the ascorbic acid contents, calculated on this basis from trichloroacetic acid extracts, of the alimentary canals of three groups of guinea-pigs. The first group, which was almost if not entirely exhausted of ascorbic acid on a scorbutic diet, received 100 mg. of ascorbic acid (synthetic)[2] by means of an injection into the jugular vein under a local anaesthetic, and the animals were

Table I.

Group	Days on scorbutic diet	Weight g.	Ascorbic acid injected mg.	Ascorbic acid in small intestine mg.		Ascorbic acid in remainder of alimentary canal mg.	
				Total	Per g.	Total	Per g.
1	7	300	100	3·0	0·30	1·5	0·10
	8	280	100	3·0	0·33	2·0	0·14
	10	300	100	3·0	0·24	2·0	0·12
	10	270	70	2·6	0·15	2·0	0·08
2	7	272	0	0·66	0·04	0·50	0·03
	8	280	0	0·40	0·03	0·25	0·01
	15	395	0	nil	—	nil	—
3	Mixed diet	580	0	5·0	0·25	1·5	0·08
	,,	575	0	6·0	0·30	2·0	0·10
	,,	615	0	3·5	0·18	1·4	0·10
	,,	260	0	2·0	0·20	1·5	0·10

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[1] Member of the Scientific Staff, Medical Research Council.

[2] Kindly supplied by Hoffmann-La Roche & Co.

killed two hours later. The second was a control group subsisting on a scorbutic diet, and the third received for a number of weeks a mixed diet containing cabbage *ad lib.* and in addition high doses of decitrated lemon juice *per os.* All the animals were killed by stunning and bleeding. There can be little doubt that the rapid reduction of the indophenol in the case of the injected animals was due to ascorbic acid. It is unlikely that the injected substance has either produced from precursors in the tissues, or has been itself converted into, an inactive reducing substance, especially as it has been shown by biological tests [Johnson and Zilva, 1934] that ascorbic acid passes through the organism without change. The animals in group I were killed two hours after the injection, since it was found that by this time an equilibrium was established in the ascorbic acid content of the intestines of these guinea-pigs. It is seen from the Table that the concentration of the vitamin in the alimentary canal is high, especially in the small intestine. This will be appreciated more when it is stated that the average concentration per g. in the liver of the above guinea-pigs on the mixed diet was 0·25 mg. Furthermore the concentration in the intestine is about the same as that observed by Harde and Wolff for the mouse (0·18–0·37 mg. per g.). These authors state, on the other hand, that the concentrations in the two cases differ appreciably ("sensiblement") but give no experimental details for the guinea-pig. They are of the opinion that "il y a une différence fondamentale entre l'élaboration des produits réducteurs dans l'intestin du cobaye et de la souris."

It is perhaps superfluous to add that the above figures (groups 1 and 3) are significant since the titrations were carried out on extracts equivalent to about 2 g. of tissue which usually reduced quickly over 6 ml. of $N/1000$ indophenol in the case of the small intestine. On the other hand the figures obtained in group 2, the control group on the scorbutic diet, are so low that they might be due to very small quantities of ascorbic acid, as given in the Table, or to the presence in higher concentration of "slow" reducing substances such as cysteine.

It therefore emerges from this experiment that when guinea-pigs are on a diet containing ascorbic acid the substance is selectively absorbed by the tissues of the intestinal tract, principally by those of the small intestine. It is above all plain from my results that this accumulation cannot be due to the passage of the vitamin of the ingested food through the gut, since when the ascorbic acid was injected into the blood stream, concentrations of ascorbic acid of an order similar to that obtained with guinea-pigs on a mixed diet were recorded. The guinea-pig, then, resembles the rat and the mouse, which are capable of synthesising ascorbic acid, in concentrating this compound in the same organs and glands, *viz.* the anterior lobe of the pituitary, the adrenals, the liver, the intestine, *etc.* when the vitamin is adequately supplied *per os* or by injection into the blood stream.

REFERENCES.

De Caro (1934). *Z. Physiol. Chem.* **223**, 229.
Giroud and Leblond (1934). *Bull. Soc. Chim. Biol.* **16**, 1352.
Harde and Wolff (1934). *Compt. Rend. Soc. Biol.* **116**, 288.
Hopkins (1934). *Chem. Ind.* **53**, 874.
Johnson and Zilva (1934). *Biochem. J.* **28**, 1393.

XIV. THE CHEMICAL COMPOSITION OF TEETH.

I. THE ESTIMATION OF FLUORINE AND THE FLUORINE CONTENT OF NORMAL TEETH.

By JOAN HYLTON BOWES and MARGARET MARY MURRAY.

From the Department of Physiology, Bedford College, University of London.

(*Received November 24th, 1934.*)

This investigation is part of a general consideration of the composition of dental enamel. The main object is to determine the composition of teeth of good structure, so that later the question of the relationship between chemical constitution and hypoplasia may be considered. The term hypoplasia is used in the sense intended by Mellanby [1923; 1934], who established a correlation between surface texture and histological structure, and the hypoplasia therein exhibited, with susceptibility to caries.

It is fairly generally agreed that the calcium phosphate of teeth and bones is not present as tertiary calcium phosphate, but as a phosphate of the apatite type $3Ca_3(PO_4)_2.CaX.$, in which X can be F_2, Cl_2, $(OH)_2$ or CO_3. Gassmann [1910] suggested carbonato-apatite and Bassett [1927] hydroxyapatite as the bone compound. Robison and Soames's [1925] results on bone support the idea that the chief constituent is an apatite. In a general consideration of the results of various workers for the calcium and phosphorus contents of bones and teeth Robison [1932] came to the same conclusion.

Of recent years bones and teeth have been subjected to X-ray analysis by de Jong [1926], Taylor and Sheard [1929], Roseberry *et al.* [1931], Thewlis [1932], Klement and Tromel [1932] and Bredig [1933]; all have concluded that the chief mineral present is an apatite. The application of this method has so far not been able to settle the question of the particular form of apatite.

Fluoroapatite is geologically common, but other isomorphous forms exist and have been considered in relation to the substance in bone and teeth by Hendricks *et al.* [1932]. The occurrence of fluorine in teeth has been investigated by many workers, but there does not seem to be any generally accepted idea as to the quantity of this element present. The reason is probably the difficulty of estimation. Several textbooks give values of the order of 0·3 % fluorine. The fluorine content of fluoroapatite is theoretically 3·77 %. No recent determinations on teeth have given values of this order of magnitude, so obviously the inorganic substance of teeth is not all fluoroapatite.

Fluorine is not an essential element in the diet. Sharpless and McCollum [1933] proved that fluorine-free diets were better for teeth formation, in rats, than diets containing relatively small amounts, *e.g.* 0·005 %, of fluorine. The occurrence of fluorine in drinking waters, to the extent of more than about two parts per million, is now recognised as the cause of that condition known as "mottled teeth." Such teeth show either a dull opaque chalky appearance or characteristic unsightly brown stains on the enamel. A more detailed description is to be found in a paper by Ainsworth [1933]. Extensive investigations have been carried out in America by McKay, originating in the work of Black and McKay [1916] and reported in the numerous publications of McKay and his collaborators on the subject of mottled teeth. Independently since 1930 Smith *et al.* [1931] have published the results of investigations which have led to the association of fluorine in drinking water to the extent of more than 1·5 parts per million with the occurrence of mottled teeth. Churchill [1931] proved this connection by determination of the fluorine content of the waters.

The effects of feeding sodium or calcium fluoride to animals had been observed by several earlier investigators, notably Sonntag [1916]. McCollum *et al.* [1925] revived the interest in this subject and reported that the inclusion of sodium fluoride in the diet of rats caused marked changes in the growth and appearance of the incisor teeth.

The danger of feeding rock phosphate, which frequently contains fluoroapatite, has been well established by Velu [1931]. He found that natural fluorophosphates fed to rats caused marked diminution of food intake; the animals did not thrive and many died. This condition of chronic fluorosis he called fluoride cachexia. Bone changes resembling osteomalacia have been observed by Christiani and Gautier [1925] in animals living on vegetation in the neighbourhood of chemical works which liberate fluorine, or certain of its compounds, into the atmosphere.

In this country an investigation of the teeth of persons living in Maldon, Essex, by Ainsworth [1933] revealed that mottled teeth are endemic there. The water of Maldon was found at that time to contain 4·5 to 5·5 parts of fluorine per million.

But in spite of this interest in the effects of fluorine, few determinations of the fluorine content of teeth have been made within recent years, so that there have been no definite conclusions as to whether the fluorine affects the deposition of the normal constituents of teeth or whether it enters into the composition of mottled teeth.

As a preliminary to the investigation of this problem it was necessary to find a reliable method for the estimation of fluorine in small amounts of material and suitable for very small percentages of the element. It is with this problem that the present communication deals; it is hoped that later a series of determinations of the fluorine content of the teeth of animals on fluorine-containing diets and of mottled teeth will be possible.

The estimation of fluorine in dental material, with its large proportion of calcium phosphate, is a matter of some difficulty. The earlier workers usually employed volatilisation as hydrofluoric acid or as silicon tetrafluoride, in combination with the glass etching test. Gassmann [1910] using this method found no fluorine in bones or teeth, but Gautier and Clausmann [1913] found 0·02 to 0·056 % in bones and 0·062 % in teeth. Mayrhofer *et al.* [1932] used the silicon tetrafluoride procedure combined with indirect determination of the fluorine by colorimetric estimation of the silicon. This later step is unwise, particularly since the gas was caught in caustic soda in a glass vessel. In the determination of silicon only platinum or "bakelite" vessels should be used. Sharpless and McCollum [1933] used the silicon tetrafluoride method for bones; their determination is referred to later. A method described by Willard and Winter [1933], involving treatment with perchloric acid, in the presence of glass beads, thereby converting the fluorine into hydrofluorosilicic acid, followed by thorium nitrate titration of the fluorine with zirconium alizarinate as an indicator, seems to have much to commend it. Preliminary trials on a known calcium phosphate-sodium fluoride mixture were not satisfactory with regard to the volatilisation. It is to be noted that probably one gram of tooth material contains approximately 0·2 mg. fluorine, so that the determination would be close to the limit of accuracy of the method. About 30 rats' incisors are required to give 1 g. The success of the volatilisation methods seems to depend very much on the amount present, being less reliable for small quantities.

Other methods which have been used include that of Meyer and Schulz [1925; 1926]. This involves precipitation as a lanthanum fluoride-acetate complex. Trebitsch [1927] used this and found 0·29 to 0·59 % in tooth crowns. When we applied this method to a known fluorine-containing powder it proved quite unreliable; the results were too high, as we believe those of Trebitsch to be.

The next method tried was Mervin's [1909] pertitanate method. When fluorides are added to a solution of a pertitanate, made by addition of hydrogen peroxide to a solution of titanic sulphate in sulphuric acid, bleaching of the orange-yellow colour occurs. The extent of the bleaching is compared with that caused by a standard. Since this work has been in progress, Sharpless and McCollum [1933] have used a modification of the titanium method for the fluorine content of bones.

The pertitanate method is certainly more reliable than the lanthanum method, but it is not very sensitive and so is not ideal when small quantities of material only can be obtained. Estimations by this method were carried out in the following way. Approximately one gram of powdered enamel was fused with sodium carbonate in a platinum crucible. The melt was extracted with successive quantities of hot water, a considerable volume being used. The solution was filtered, concentrated and partially neutralised by the addition of $4N$ sulphuric acid in presence of an aqueous solution of phenolphthalein, care being taken to avoid excess of acid. Excess silver sulphate was then added to remove phosphates and the precipitate filtered off and washed. The excess of silver was removed by addition of sodium chloride, the solution filtered, the precipitate washed and the filtrate brought to a known volume by evaporation. Several 100 ml. graduated flasks were then taken and to each were added 10 ml. 65 % by volume sulphuric acid, 10 ml. of the titanic sulphate solution, made according to the directions of Warren *et al.* [1925], 3 ml. 10 volume hydrogen peroxide and then either a known volume of the unknown fluoride or a known volume of a comparable standard sodium fluoride solution. The solutions were allowed to stand and then were compared in a colorimeter. The values obtained for human enamel by this procedure varied from nothing to 0·04 %, the most usual value being 0·01 %. Ten estimations in all were made[1]. We then tested the sensitivity of this method and considered that the low results might be due to its lack of sensitivity. Sharpless and McCollum [1933] obtained less than 0·04 % fluorine in the bones of rats fed on a diet containing very little fluorine, *i.e.* 0·001 % of the ration.

The estimation of fluorine in sea-water and in the waters of districts where mottled teeth are endemic has of recent years been made by a method originally introduced by de Boer [1924] and latterly used and modified by Casares and Casares [1930].

The principle of the method is that solutions of zirconium salts, whether nitrate or oxychloride, and sodium alizarinsulphonate form a red lake, stable in acid solution. The coloured lake is slowly bleached by fluorine, the bleaching being proportional to the fluorine concentration, hence by colorimetric comparison with a standard fluoride solution, an estimation can be made.

Taylor and Thompson [1933] applied this method to sea-water and Elvove [1933] to drinking water. In our determinations we first used the Taylor and Thompson directions for making the lake, but later we employed the Elvove [1933] solutions.

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[1] We are indebted to Miss M. O. Barrie for these titanium method determinations.

The estimation of fluorine in teeth by this method was complicated by the presence of other substances tending to cause precipitation of the lake. Taylor and Thompson, in their report on the estimation of fluorine in sea-water, state that chlorine and sulphate ions have bleaching actions, but that phosphate and calcium ions do not interfere. It was found however that the concentration of calcium and phosphate ions in our solutions of tooth material definitely caused precipitation of the lake. Samples of sea-water and London tap-water also showed precipitation after 18 hours. Various methods for removing interfering substances were tried. Firstly phosphate precipitation was effected with calcium chloride and ammonia. This procedure when checked on fluoroapatite gave a value of 4·733 % fluorine, which was known to be too high. When greater care was taken to remove ammonia the result was nearer the correct value but still there was a tendency to precipitation. We next tried precipitation of interfering substances by excess of zirconium nitrate, but though this removed the source of precipitation of the lake, excess zirconium salt prevented the bleaching action of fluorides.

Finally we used precipitation by silver nitrate and removal of excess silver by sodium chloride. In many of these preliminary tests, a known fused mixture of calcium phosphate and calcium fluoride was employed in place of enamel. We always determined the chlorine content of our solutions and adjusted the chlorine content of the standard fluorine solutions to be the same. Finally we determined the nitrate content by calculation and adjusted this by addition of sodium nitrate to the standards.

In all these methods, the resulting solution was brought to a known volume, and an aliquot part taken, and to it were added 2 ml. 6*N* HCl and 2 ml. of the zirconium lake solution used by Taylor and Thompson. The solution was then brought to the boiling-point and left for several hours. At the same time solutions containing different standard amounts of sodium fluoride and having the same chlorine and nitrate contents were treated similarly.

The values obtained for the fluorine of human enamel by these procedures were as follows:

Method	Fluorine %
Lanthanum method	Unreliable values
Titanium method	0·0 to 0·04
Zirconium-alizarin method with calcium chloride precipitation	0·093
Zirconium-alizarin method with calcium chloride precipitation and removal of ammonia	0·06
Zirconium-alizarin method, silver precipitation, chlorine content adjusted	0·07
Zirconium-alizarin method adjusted for chlorine and nitrate concentrations	0·02

We realised throughout the determinations that the tooth solutions might contain small traces of substances capable of affecting the colour of the lake and we therefore considered it necessary to make certain that we were estimating fluorine by taking a sample of enamel and treating a portion of it with sulphuric acid to drive off the fluorine as hydrofluoric acid.

This experiment was a crucial one and will be described in detail. Two portions of ashed human enamel, 1·1684 g. each, were taken and fused with 6 g. Na_2CO_3. The melts were extracted very thoroughly with boiling water. One sample B was evaporated to dryness, H_2SO_4 added and the whole heated cautiously to drive off the HF. The residue was extracted with water and solid Na_2CO_3 added to neutralise; the resulting solution was the "blank." This solution and the solution of the other melt, solution A or the "unknown," were each brought to a volume of about 100 ml. and both were treated in the following way. 60 ml. of a strong solution of $AgNO_3$ were added, the precipitates filtered off in the dark and washed. The excess silver was removed by addition of a solution of NaCl and the precipitates filtered off and washed. The resulting filtrates were brought to a known volume and the chlorine concentration of each determined by titration with standard $AgNO_3$, so that later, the chlorine contents of the solutions could be equalised and that of the standard fluorine made the same.

Sulphate ions interfere with the determination; sulphate was therefore removed from B by the addition of the calculated quantity of $Ba(NO_3)_2$, after determination of sulphate. After filtration and concentration, the solution was tested for sulphate and barium; a very small amount of sulphate was present. A trace of Na_2SO_4 was added to A and to the fluoride standards to equalise the sulphate throughout.

The two solutions A and B were then adjusted to the same chlorine content, so that they finally contained equal amounts of their different constituents except fluorine. A solution C was made having the same concentration of NaCl, $NaNO_3$ and Na_2SO_4 as A and B; this served for the making of the standard fluoride solutions. 25 ml. portions of solutions A and B were taken and to them were added HCl and the zirconium-alizarin lake; the solutions were brought to the boil. At the same time, portions of solution C to which were added varying amounts of NaF standard solution were similarly treated. All the volumes were made equal and the solutions matched in Nessler tubes. We found it better to find an exact match for the unknown solutions rather than to compare them in a colorimeter because there is a change of tint as well as intensity.

The unknown solution was definitely bleached as compared with the acid-treated blank. The amount of fluorine in the unknown was determined by finding a fluorine standard which compared with it. It was found that 2 ml. of the standard sodium fluoride (1 ml. = 0·01 mg.) gave slightly less bleaching, while 3 ml. of the standard gave definitely more bleaching.

$$\begin{aligned} &\text{Hence 25 ml. A} &&\equiv 0{\cdot}025 \text{ mg. F} \\ &\therefore \text{250 ml. A} &&\equiv 0{\cdot}25 \quad \text{,,} \\ &\%\ \text{Fluorine} &&\equiv \frac{100 \times 0{\cdot}25}{1168{\cdot}4}\% \\ & &&\equiv 0{\cdot}0214\ \%. \end{aligned}$$

Another comparison was made by taking the acid blank B and adding fluoride to it. When 2 ml. were added there was too little bleaching and when 3 ml. were added too much bleaching, as compared with the unknown. Hence this procedure gave the same answer as before. (It is of interest to note that this fluorine value indicated that the teeth contained 0·51 % fluoroapatite.)

The process is a lengthy one because of the precautions necessary to eliminate precipitation effects and alteration of dye colour by substances other than fluorine.

Recently we have carried out determinations by the titanium method and the zirconium-alizarin method on the same sample of rats' teeth, the results were:

Titanium method	0·034 %,
Zirconium method	0·030 %.

That the latter is more sensitive is shown by the fact that for this method only 20 ml. of the tooth solution were used and matched against a standard solution containing 0·06 mg. fluorine per 100 ml., whereas for the titanium method, 75 ml. solution were taken and matched against a standard containing 2 mg. per 100 ml.

We are at present applying the zirconium method to the estimation of the fluorine content of teeth of rats under various experimental conditions and to mottled teeth.

It has become clear during this investigation that normal teeth, that is non-mottled, may vary in their fluorine content within certain limits.

SUMMARY AND CONCLUSIONS.

1. Methods used in recent years for the estimation of small quantities of fluorine have been reviewed.
2. The zirconium-alizarin method has been applied to solutions of tooth material.
3. The quantity of fluorine in human enamel of the London area was 0·02 %.
4. The teeth of rats fed on diets, not designed to be fluorine-free, contained 0·02 % to 0·03 % fluorine.

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REFERENCES.

Ainsworth (1933). *Brit. Dent. J.* **55**, 233.
Bassett (1917). *J. Chem. Soc.* **111**, 620.
Black and McKay (1916). *Dental Cosmos.* **58**, 129.
de Boer (1924). *Chem. Weekblad.* **21**, 404.
Bredig (1933). *Z. physiol. Chem.* **216**, 239.
Casares and Casares (1930). *Ann. Re. Soc. Esp. Fisol. Quim.* **28**, 1189.
Christiani and Gautier (1925). *Compt. Rend. Soc. Biol.* **92**, 139.
Churchill (1931). *Ind. Eng. Chem.* **23**, 996.
Elvove (1933). *Public Health Rep. U.S.A.* No. 48.
Gassmann (1910). *Z. physiol. Chem.* **70**, 161.
Gautier and Clausmann (1913). *Compt. Rend. Acad. Sci.* **156**, 1425.
Hendricks, Jefferson and Mosley (1932). *Z. Krystall.* **81**, 352.
de Jong (1926). *Rec. Trav. Chim. Pays Bas* **45**, 445.
Klement and Tromel (1932). *Z. physiol. Chem.* **213**, 263.
McCollum, Simmonds and Becker (1925). *J. Biol. Chem.* **63**, 553.
Mayrhofer, Schneider and Wasitsky (1932). *Biochem. Z.* **251**, 70.
Mellanby (1923). *Brit. Dent. J.* **44**, 1.
—— (1934). *Med. Res. Council Spec. Report.* No. 191.
Mervin (1909). *Amer. J. Sci.* **28**, 119.
Meyer and Schulz (1925). *Z. angew. Chem.* **38**, 203.
—— —— (1926). *Z. anal. Chem.* **69**, 465.
Robison (1932). Significance of phosphoric esters in metabolism. (New York Univ. Press.)
—— and Soames (1925). *Biochem. J.* **19**, 153.
Roseberry, Morse and Hastings (1931). *J. Biol. Chem.* **90**, 395.
Sharpless and McCollum (1933). *J. Nutrition,* **6**, 163.
Smith, Lantz and Smith (1931). *Tech. Bulletin Univ. Arizona, College of Agriculture,* No. 32.
Sonntag (1916). *Chem. Zentr.* **8**, 1095.
Taylor and Sheard (1929). *J. Biol. Chem.* **81**, 479.
—— and Thompson (1933). *Ind. Eng. Chem.* **5**, 87.
Thewlis (1932). *Brit. J. Radiology,* **52**, 353.
Trebitsch (1927). *Biochem. Z.* **191**, 234.
Velu (1931). *Compt. Rend. Soc. Biol.* **108**, 750.
Warren, Gimingham and Page (1925). *J. Agric. Sci.* **15**, 516.
Willard and Winter (1933). *Ind. Eng. Chem. Anal.* **5**, 7.

XV. THE REDUCTION OF GLUTATHIONE BY THE WARBURG-CHRISTIAN SYSTEM.

By the late NORMAN URQUHART MELDRUM[1]
and HUGH LEWIS AUBREY TARR[2].

From the Biochemical Laboratory, Cambridge.

(*Received November 3rd, 1934.*)

Hopkins [1921] suggested that the physiological significance of glutathione might lie in its ability to function as transporter of hydrogen from oxidisable molecules in the cell to molecular oxygen. His observation that certain animal tissues were able to reduce the oxidised form of the peptide, and that the reduced form was autoxidisable, lent support to this view. Furthermore he recognised that glutathione apparently always occurs in the reduced form in animal tissues.

Hopkins and Dixon [1922] discovered that "succinoxidase" was quite unable to reduce glutathione. Elliott [1928] confirmed this observation and showed moreover that the "xanthine oxidase" system would not reduce the oxidised peptide. Hopkins and Elliott [1931] studied the behaviour of glutathione in the freshly excised, chopped livers of various animals and found that livers from well-fed animals reduced glutathione more strongly than did those from starved animals. That certain definite "dehydrogenase" systems were involved in the reduction of glutathione in this tissue was strongly suggested by the fact that much of the reducing power was destroyed by heating liver for an hour at 52°. Their results proved definitely that glutathione could function as oxygen carrier in living cells. The exact nature of the system or systems which reduced glutathione in this tissue was not ascertained.

Shortly afterwards Mann [1932] discovered that the glucose dehydrogenase of liver reduced glutathione under anaerobic conditions, and almost simultaneously Meldrum [1932] observed that intact mammalian erythrocytes possessed some system capable of reducing the oxidised peptide in the presence of glucose. Apparently no other systems in living cells capable of reducing glutathione have been discovered, though it is known that certain bacteria reduce the peptide by virtue of their natural metabolites [Tarr, 1933]. The present work has shown that the isolated dehydrogenase system of Warburg and Christian strongly reduces glutathione.

Experimental.

The barium hexosemonophosphate "natural" ester used in these experiments was obtained from the British Drug Houses. The barium hexosemonophosphate "synthetic" ester was prepared by the method of Levene and Raymond [1931], and the corresponding barium phosphohexonic acid by oxidation of the synthetic ester with bromine [Robison and King, 1931]. Barium diphosphoglycerate was made by the method of Jost [1927] and barium fructosediphosphate was kindly given to us by Prof. Keilin. All these compounds gave analyses for barium and phosphorus in good agreement with theory. The specific rotations and "aldose" and "fructose" titres corresponded closely with the values recorded in the literature except in the case of the "synthetic" ester which had a low specific rotation and gave a definite "Selivanoff test" for fructose [Macleod and Robison, 1929]. However, since the "synthetic" ester proved almost as active as the "natural" ester, it was employed in many of the following experiments.

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[1] Late Beit Memorial Research Fellow.

[2] Some time 1851 Overseas Scholar and External Research Student, Emmanuel College, Cambridge; now Research Bacteriologist, Rothamsted Experimental Station, Harpenden, Herts.

Crude co-enzyme powder was prepared from horse erythrocytes, and from this the solution was made [Warburg and Christian, 1931]. In the Tables "mg. of co-enzyme" signifies that an amount of solution corresponding to the given weight of co-enzyme was used. The enzyme of the reducing system was prepared from rat blood [Warburg and Christian, 1931] and also from top brewery yeast and from baker's yeast [Warburg and Christian, 1932]. Experience showed that crude co-enzyme prepared as above frequently, though not always, contained fairly large amounts of a substance which, on reduction, gave a strong nitroprusside test (probably glutathione), and for this reason fairly high "iodine titres" were frequently obtained in control experiments[1].

Oxidised glutathione was prepared according to Pirie [1930; 1931], solutions being adjusted to p_H 7·4 with N NaOH before use. The amount of reduced glutathione present in experimental solutions was estimated by titrating with $N/100$ iodine after sufficient potassium iodide (25 %) had been added to bring the final concentration of this salt to 1 %. Starch was employed as indicator.

The reduction of glutathione by Warburg and Christian's reducing system in the presence of various substrates.

1. *Synthetic hexosemonophosphate, fructosediphosphate and glucose as substrates.* The substances noted in Table I were added to 75 ml. Thunberg tubes, the final volume of fluid in each tube being adjusted to 20 ml. with distilled water. 0·05 ml. of caprylic alcohol was added to each tube before evacuation at a water-pump, this substance having the dual virtue of functioning as a germicide and foam preventer. The evacuated tubes were incubated for 6 hours in a water-thermostat at 37°, and then a nitroprusside test (1 ml. used) and iodine titration were made on the contents in each instance. In Table II the results of this experiment are recorded. It is apparent that reduction of glutathione is not effected by the enzyme or co-enzyme alone in the absence of hexosemonophosphate, or by the complete system when heated. Rapid and

Table I.

	Thunberg tube No.						
Reagent	1	2	3	4	5*	6	7
$M/5$ phosphate buffer, p_H 7·4 (ml.)	5	5	5	5	5	5	5
Oxidised glutathione (mg.)	20	20	20	20	20	20	20
Synthetic barium hexosemonophosphate (mg.)	40	0	40	40	40	0	0
Fructosediphosphate (used as neutral sodium salt) (mg.)	0	0	0	0	0	0	40
Glucose (mg.)	0	0	0	0	0	40	0
Enzyme (from rat blood) (ml.)	2	2	0	2	2	2	2
Co-enzyme (mg.)	100	100	100	0	100	100	100

* Absolute control, heated 5 minutes in a boiling water-bath.

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[1] Warburg and Christian [1934] have recently stated that Nagelein has discovered that their "co-ferment II" is merely a mixture of "co-ferment I" and glutathione.

Table II.

	Tube No.						
	1	2	3	4	5	6	7
Nitroprusside test	+ + + +	–	–	–	–	–	+
ml. of $N/100$ iodine required	6·7	0·5	0·2	0·5	0·4	0·5	0·9
*Reduced glutathione in mg.	19·0	–	–	–	–	–	1·9

* Calculated from the iodine titre after subtracting the control.

practically quantitative reduction of the glutathione occurred in the presence of the complete system and hexosemonophosphate. Fructosediphosphate acted only as a very feeble donator of hydrogen in this system and glucose was not utilised at all. Unfortunately a pure sample of Neuberg's fructosemonophosphate was not obtained; crude samples obtained by the hydrolysis of fructosediphosphate (candiolin) were found to be only slightly more active than fructosediphosphate itself.

2. *Natural hexosemonophosphate, synthetic hexosemonophosphate, phosphohexonic acid and diphosphoglyceric acid as substrates.* The relative values of the above compounds as donators of hydrogen in the reducing system under survey were next determined. The technique was generally identical with that used in the previous experiment. Each Thunberg tube contained 2 ml. of $M/5$ phosphate buffer p_H 7·4, 50 mg. of co-enzyme, 1 ml. of enzyme (from rat blood), 50 mg. of oxidised glutathione and, with the exception of the controls, 10 mg. of the substrate under investigation. All substances were tested in duplicate, and titrations were made at the end of 12 and 24 hours at 37°. The results, which are given in Table III, show that, while both natural and synthetic hexosemonophosphoric acids are readily activated, the corresponding phosphohexonic acid is less readily attacked, and diphosphoglyceric acid is not activated at all. The reduction was almost always complete after 12 hours: whether this was due to a complete utilisation of all available substrate, or to exhaustion of enzyme was not determined.

Table III.

	Time in hours			
	12		24	
Substrate	ml. $N/100$ iodine	mg. of GSH*	ml. $N/100$ iodine	mg. of GSH*
Controls (no substrate added)	0·6	0	0·6	0
Natural barium hexosemonophosphate	9·4	26·6	10·0	28·5
Synthetic barium hexosemonophosphate	6·9	19·1	7·2	20·0
Barium phosphohexonate	4·1	10·6	4·1	10·6
Barium diphosphoglycerate	0·6	0	0·6	0

* The reduced glutathione was calculated from the iodine titre after the controls were subtracted.

Glutathione as oxygen carrier in the Warburg-Christian reducing system.

The oxidation of hexosemonophosphoric acid by the Warburg-Christian reducing system with various concentrations of glutathione as oxygen carrier was studied employing Barcroft differential manometers. In all manometric experiments the temperature was 37° and the gas space was filled with air. Four manometers were used, and the amounts of reagents employed in each are recorded in Table IV. The final volume of liquid in each manometer cup was

Table IV.

	Manometer No.							
	I		II		III		IV	
Reagent	*L	*R	L	R	L	R	L	R
Enzyme (from rat blood) (ml.)	0	1	0	1	0	1	0	1
Co-enzyme (mg.)	0	50	0	50	0	50	0	50
Oxidised glutathione (mg.)	0	0	0	10	0	20	0	40
Barium hexosemonophosphate (synthetic) (mg.)	0	40	0	40	0	40	0	40
$M/5$ phosphate buffer, p_H 7·4 (ml.)	1	1	1	1	1	1	1	1

* L and R refer to right- and left-hand manometer cups.

made up to 5 ml. with distilled water and 0·05 ml. of caprylic alcohol added as germicide. In the centre tubes filter-papers moistened with 6 % KOH were employed to absorb CO_2 [Dixon and Elliott, 1930]. The manometers were equilibrated for 10 minutes before the taps were closed, and readings were then taken over 10 hours at this temperature. The results are given in Fig. 1. It is evident from this diagram that the addition of glutathione caused a considerable increase in oxygen uptake, the increase in oxidation velocity being approximately proportional to the amount of glutathione added. In no case was the oxygen uptake very rapid, and in no case comparable with that obtained by Warburg and Christian [1931; 1932; 1933] with the same reducing system when either methylene blue or yellow pigment was employed as carrier of oxygen. Perhaps a greater activity of the enzyme preparations employed by these workers accounted in part for this increased velocity. It is possible that the presence of a trace of cysteine would have accelerated the uptake in the case of glutathione experiments [Meldrum and Dixon, 1930]. At the conclusion of the experiment the fluid in each right-hand manometer cup was titrated with $N/100$ iodine solution. The results given in Table V show that the reducing system was powerful enough to maintain practically the whole of the glutathione present in the reduced form even under the strongly aerobic conditions of the experiment. Perhaps the

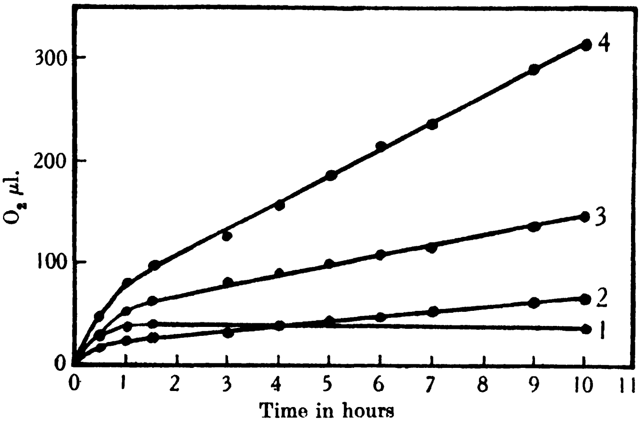

Fig. 1. The effect of glutathione on the oxidation of hexosemonophosphate by the Warburg-Christian reducing system.

1, Control (no added glutathione). 2, 10 mg. of GSSG added. 3, 20 mg. of GSSG added. 4, 40 mg. of GSSG added.

Table V.

Manometer No.	ml. of $N/100$ iodine	mg. of GSH*	mg. of GSSG originally added
I	1·5†	0	0
II	4·7	9·7	10·0
III	7·6	18·5	20·0
IV	11·6	30·0	40·0

* Calculated from iodine titre after control subtracted.

† High figure due to —SH in control (from co-ferment).

presence of reducing systems such as this in yeast may account for the results obtained during some studies of the glutathione content of yeast under various conditions [Meldrum, 1930], in which it was found that practically the whole of the glutathione present remained in the reduced form even when aerated and in the presence of cyanide or narcotics.

Cysteine as oxygen carrier in the Warburg-Christian reducing system.

This experiment was run upon similar lines to the previous one, the manometric set up being identical with the exception that cysteine (added as the neutralised hydrochloride) was used in place of glutathione. In this experiment ordinary cysteine, and not cysteine freed from traces of iron, was used. The course of oxidation of the hexosemonophosphate at 37° was followed and the results are plotted graphically (Fig. 2). During the first hour and a half of the experiment in the case of the manometers with added cysteine the oxygen

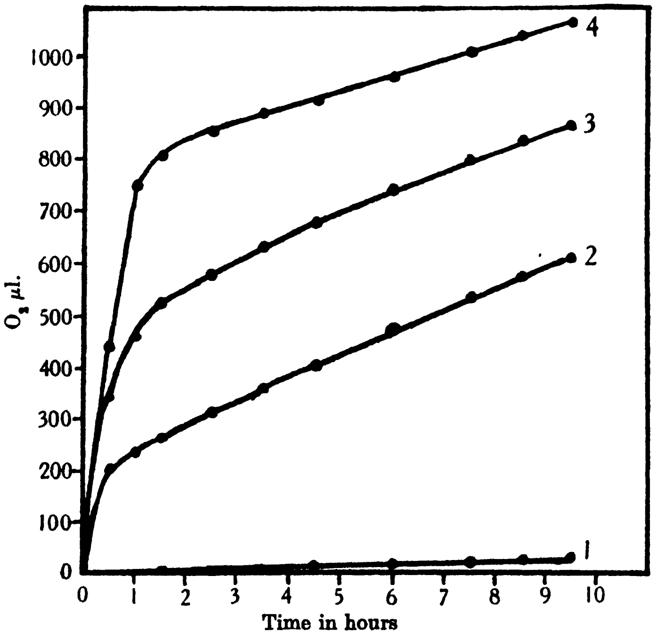

Fig. 2. The effect of cysteine on the oxidation of hexosemonophosphate by the Warburg-Christian reducing system.

1, Control (no added cysteine). 2, 10 mg. of cysteine added. 3, 20 mg. of cysteine added. 4, 40 mg. of cysteine added.

uptake was extremely rapid, this undoubtedly being primarily due to the oxidation of cysteine to cystine. Observation showed that cystine precipitated in flasks to which cysteine had been added. Subsequently the velocity of oxidation became fairly steady and was practically identical in the three experiments in which cysteine was added. However the velocity of oxidation in all three cysteine experiments was considerably greater than in the control without cysteine. At the conclusion of the experiment the amount of —SH present was determined as in the previous experiment, the results being recorded in Table VI. It is

Table VI.

Manometer No.	Nitroprusside test	ml. of $N/100$ iodine required
I	+ + +	1·5
II	–	2·5
III	–	2·6
IV	–	2·7

evident that practically all the cysteine had been oxidised, and had remained in this form, and it appears therefore that the factor limiting the velocity of oxidation in this instance was the velocity of reduction of the cystine, and not, as in the case of glutathione, the velocity of re-oxidation of the reduced form. The fact that no nitroprusside test was obtained in the cystine-containing manometers suggests that the cystine causes the oxidation of the small amount of glutathione present in the co-ferment.

The velocity of the anaerobic reduction of glutathione.

75 ml. Thunberg tubes were employed as usual. Eight tubes each containing 1 ml. enzyme solution, 50 mg. of co-enzyme, 10 mg. of barium hexosemonophosphate, 1 ml. of $M/5$ phosphate buffer p_H 7·4 and 30 mg. of oxidised glutathione were prepared, and two identical tubes without glutathione as controls. The final volume of liquid in each tube was made up to 6 ml. with distilled water, and each was evacuated, incubated and the amount of —SH present determined in the usual manner, estimations being made at intervals in order to obtain an

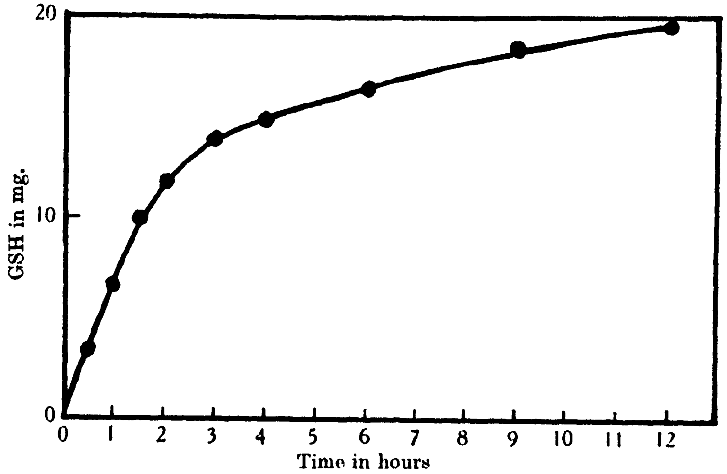

Fig. 3. The velocity of the anaerobic reduction of glutathione by the Warburg-Christian system.

idea of the velocity of reduction. At the end of 12 hours controls without added glutathione gave an average iodine titre of 1·7 ml. of $N/100$ solution, this being due to the —SH present in the co-ferment. In Fig. 3 the absolute —SH production (calculated as glutathione) in the series of eight tubes over a 12-hour period is shown. The reduction was rapid at first and then fell off, and at the end of the time only about 60 % of the glutathione was reduced. It was not determined whether this was due to exhaustion of enzyme or to exhaustion of substrate.

The reduction of glutathione employing yeast enzyme.

In this case the enzyme of the reducing system prepared both from brewer's top yeast and from baker's yeast was employed in place of enzyme made from rat's blood. The method of studying the reduction of glutathione was identical with that described in previous experiments, the final volume of fluid in each tube being 10 ml. The set up of the experiment is shown in Table VII. The results given in Table VIII show that an active enzyme is obtainable from top yeast and that it is inactive with glucose and fructose in place of hexosemonophosphoric acid as substrate.

Table VII.

	Tube No.					
Reagent	1	2	3	4	5	6
Oxidised glutathione (mg.)	20	20	20	20	20	20
Enzyme (from brewer's top yeast) (ml.)	2	0	2	2	2	2
Co-enzyme (mg.)	50	50	0	50	50	50
Barium hexosemonophosphate synthetic (mg.)	40	40	40	0	0	0
Glucose (mg.)	0	0	0	0	40	0
Fructose	0	0	0	0	0	40
$M/5$ phosphate buffer, p_H 7·4 (ml.)	1	1	1	1	1	1

Table VIII.

Tube No.	Nitroprusside test	ml. of $N/100$ iodine required	Estimated GSH in mg.
1	+ + + +	6·3	18·8
2	–	0·1	0
3	–	0·1	0
4	–	0·1	0
5	–	0·1	0
6	–	0·1	0

An exactly similar experiment was run using enzyme from baker's yeast, only the experiments with fructose and glucose were omitted. Practically identical results were obtained, the iodine titre in the presence of hexosemonophosphate being 5·3 ml. of $N/100$ solution, which corresponds to 16 mg. of reduced glutathione.

DISCUSSION.

The results of these experiments are of some interest in that they suggest that the hexosemonophosphate system may account for a considerable fraction of the reducing power of cells toward glutathione. Unpublished observations by one of us (H. T.) have shown that Harrison's glucose dehydrogenase preparation [Harrison, 1933] is able to reduce glutathione in the presence of hexosemonophosphoric acid as substrate, and this suggests that a similar system exists in liver. It is possible that such a system was responsible for some of the reduction of glutathione by liver noted by Hopkins and Elliott [1931]. It remains to be found whether or not muscle and other mammalian tissues possess this system. Up to the present time it seems as if only those enzymes which are intimately connected with the oxidation of carbohydrates in the cell are involved in the reduction of glutathione. Whether this fact is of any significance remains to be determined.

SUMMARY.

1. Glutathione is reduced both aerobically and anaerobically by the Warburg-Christian enzyme-co-enzyme system in the presence of hexosemonophosphoric acid.

2. Phosphohexonic acid and fructosediphosphoric acid also function as donators of hydrogen in this system, the former being much less readily utilised than hexosemonophosphoric acid, and the latter being only feebly activated. Diphosphoglyceric acid, glucose and fructose are not utilised.

3. Glutathione functions as oxygen carrier when hexosemonophosphoric acid is oxidised by the Warburg-Christian system in the presence of molecular oxygen, the velocity of oxidation being roughly proportional to the amount of glutathione added. In this case the limiting factor in the oxidation velocity appears to be the rate of re-oxidation of the reduced glutathione.

4. Cystine will also function as oxygen carrier in this system, but in this case the oxidation velocity is practically independent of the cystine concentration, because the limiting factor is the rate of reduction of the cystine.

5. The oxygen uptake in the presence of glutathione is in no instance as great as that obtained by Warburg and Christian when either methylene blue or yellow pigment was employed as oxygen carrier.

6. Enzyme obtained from brewer's top yeast or from baker's yeast can be substituted for enzyme obtained from rat's blood.

The success of this work is largely due to Prof. Keilin, who originally suggested the problem, and to Sir F. G. Hopkins whose interest and suggestions were most helpful. The junior author is indebted to Dr Malcom Dixon for his kindness in reading and criticising the manuscript.

REFERENCES.

Dixon and Elliott (1930). *Biochem. J.* **24**, 296.
Elliott (1928). *Biochem. J.* **22**, 1410.
Harrison (1933). *Biochem. J.* **27**, 382.
Hopkins (1921). *Biochem. J.* **15**, 286.
—— and Dixon (1922). *J. Biol. Chem.* **54**, 527.
—— and Elliott (1931). *Proc. Roy. Soc. Lond.* B **109**, 58.
Jost (1927). *Z. physiol. Chem.* **165**, 171.
Levene and Raymond (1931). *J. Biol. Chem.* **92**, 759.
Macleod and Robison (1929). *Biochem. J.* **23**, 517.
Mann (1932). *Biochem. J.* **26**, 785.
Meldrum (1930). *Biochem. J.* **24**, 1421.
—— (1932). *Biochem. J.* **26**, 817.
—— and Dixon (1930). *Biochem. J.* **24**, 472.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1931). *Biochem. J.* **25**, 614.
Robison and King (1931). *Biochem. J.* **25**, 323.
Tarr (1933). *Biochem. J.* **27**, 1869.
Warburg and Christian (1931). *Biochem. Z.* **242**, 206.
—— —— (1932). *Biochem. Z.* **254**, 438.
—— —— (1933). *Biochem. Z.* **266**, 377.
—— —— (1934). *Biochem. Z.* **274**, 112.

XVI. THE BIOLOGICAL OXIDATION OF CARBOHYDRATES.

IV. THE PHOSPHORUS REQUIREMENTS OF PERCOLATING FILTERS.

By SAMUEL HARRY JENKINS.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden.

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The purification of sewage and certain trade effluents can be effected in percolating filters by allowing the polluting liquids to trickle through a well-aerated bed of material such as clinker, gravel *etc.*, suitably graded as regards size of particles. The first stage in the purification may be the physical retention of some of the polluting matter within the filter, but ultimately purification depends upon the breakdown and oxidation by living agencies of dissolved or precipitated organic matter. Although the biological nature of the process has long been recognised, insufficient attention has been given to a study of the conditions necessary to maintain the organisms responsible for purification in a state of maximum activity. A study of this kind should include experiments on the effect of changes in concentration of compounds of nitrogen, phosphorus and potassium on the rate of oxidation of the organic matter. Sewages of domestic origin are invariably rich in nitrogenous compounds, while they also contain fats, carbohydrates and salts of potassium and phosphorus. It is improbable, therefore, that the organisms responsible for the oxidation of domestic sewage liquors will be without any essential food material. Some waste waters containing organic matter, such as those from beet-sugar factories, may, however, have insufficient quantities of certain materials to ensure optimum rate of purification in percolating filters.

In a study of the process of biological oxidation of effluents from beet-sugar factories, Richards and Cutler [1933] have shown that the rate of oxidation of the carbohydrates in solution is influenced by the amount of available nitrogen. In view of the lack of data regarding the relationship between the supply of compounds of phosphorus and the rate of oxidation of organic matter, particularly carbohydrates, in percolating filters an investigation has been made with the objects of examining (1) the effect of phosphate on the rate and extent of oxidation of sucrose; (2) the relationship between the quantity of phosphate and the removal of nitrogen from solution; (3) the availability to micro-organisms of organic compounds of phosphorus; (4) the influence of phosphate supply on the development of an active biological film on the filter media. The results are described in the present paper. Similar work published by Barritt [1931] while these experiments were in progress will be referred to later.

EXPERIMENTAL.

Filters constructed from glass tubes containing glass medium made from broken bottles were used for the first set of tests and the results are described in Exp. 1. After the filters had been supplied for 28 days with solution containing 0·1 % sucrose, pools of liquid formed on the surface of one filter owing to a heavy growth of film in the upper portion. Choking was avoided in a second series of experiments by using glass tubes as medium instead of broken glass. Details of construction of the filters have already been described [Jenkins, 1933, 1]. The results of this second set of tests are described as Exp. 2. In Exp. 3 the results are given of a large-scale trial at a beet-sugar factory. The main purpose of the experiment was to test the effect of inoculating selected organisms on to a filter. The liquids filtered were effluents from the beet-pulp presses of the factory after treatment in a sedimentation tank to remove suspended solid matter. During the period of sedimentation some fermentation occurred and the liquids became acid.

Method of analysis. Sugars, nitrogen as ammonia, nitrite and nitrate were determined by methods described previously [Jenkins, 1933, 2]. Phosphorus in the inorganic and organic forms was determined colorimetrically by the Denigès method as modified by Parker and Fudge [1927]. In the determination of phosphorus in organic combination, however, 1 ml. of 10 % calcium acetate solution was used instead of $Mg(NO_3)_2$ for igniting with the residue left after evaporation to dryness.

Experiment 1.

Each filter consisted of four glass cylinders with perforated metal plates at the lower ends. The sections were joined together by rubber bands and could easily be disconnected when samples of the effluent from any of the upper sections were required. The filtering medium was broken glass graded $\frac{1}{16}$–$\frac{1}{4}$ in. Solutions of nutrient substances in the concentrations given below were filtered at a rate of 2 litres per day (100 gallons per cubic yard of filtering medium per day, abbreviated g.y.d.). These solutions each contained 5 parts N per 100,000 and the concentrations of P_2O_5 were 0, 0·3 and 3·0 parts per 100,000 to filters A_1, B_1 and C_1, respectively. From the 23rd day until the end of the experiment on the 150th day 0·05 g. K_2SO_4 per litre was added to the solution to ensure an excess of potassium. Ammonium bicarbonate replaced ammonium chloride as the source of nitrogen from the 68th day until the end of the experiment for a reason given later, but the amount of N added remained unchanged.

Solutions filtered from beginning of experiment until the 22nd day.

Filter	A_1 g.	B_1 g.	C_1 g.
Sucrose	1·000	1·000	1·000
NH_4Cl	0·191	0·191	0·191
$MgSO_4, 7H_2O$	0·050	0·050	0·050
$CaCl_2, 6H_2O$	0·050	0·050	0·050
$NaCl$	0·005	0·005	0·005
K_2HPO_4	Nil	0·00736	0·0736
Fe_2Cl_6	Trace	Trace	Trace
Distilled water to	1 litre	1 litre	1 litre

The results of the experiment, showing the amounts of sucrose, P_2O_5 and N left in the effluents are given in Table I.

Oxidation of sugar. A small proportion of the sugar in the solution fed to filter A_1 appeared to be oxidised during passage through the filter, but the amount did not exceed 18 % of the sugar in the nutrient solution. The filtering medium was apparently devoid of organic film but microbiological examinations made by Miss Dixon proved that a few protozoa were present. It is concluded

Table I. *Effect of phosphate on biological oxidation of sucrose and removal of nitrogen.*

Exp. 1. Glass filters and glass medium ($\frac{1}{16}$ in. to $\frac{1}{4}$ in. graded glass).

Filter A_1 received sucrose equivalent to 105·3 parts glucose per 100,000 of solution, and no P_2O_5.
Filter B_1 received the same amount of sucrose as A_1, and 0·3 part P_2O_5 per 100,000.
Filter C_1 received the same amount of sucrose as A_1, and 3·0 parts P_2O_5 per 100,000.

Parts per 100,000.

Day of exp.	P_2O_5 in effluent from filter		Sucrose in effluent from filter calculated as glucose. Amount supplied, 105·3 parts			Nitrogen in effluent from filter. N supplied, 5 parts		
	B_1 (0·3 supplied)	C_1 (3·0 supplied)	A_1	B_1	C_1	A_1	B_1	C_1
14	0·0	1·25	86·0	3·6	0·4	4·800	3·530	1·430
19	0·012	1·50	95·9	19·6	1·9	4·809	3·907	3·170
27	0·0	2·50	—	0·9	1·1	4·539	3·998	4·556
36	0·002	1·50	100·4	2·6	0·7	4·916	3·041	1·176
43	0·0	1·39	—	2·3	0·3	4·460	3·442	2·675

therefore that in filter A_1 the absence of phosphate almost inhibited the growth of film so that not more than 18 % of a 0·1 % solution of sugar could be oxidised.

The addition of 0·3 part P_2O_5 per 100,000 as potassium phosphate to filter B_1 had a marked effect on the amount of sugar oxidised. When the first analyses were made after the filters had been in operation for 13 days 96·6 % of the sugar was being oxidised and over the first 43 days of the experiment the average amount decomposed was 94·5 %. Although the percentage oxidation of sugar was high the amount of film in B_1 was not considerable and consisted of a thin slime covering the glass medium in the uppermost sections, with much less in the three lower sections. The greatest contribution towards the total amount of sugar oxidised was made by section 1, which alone accounted for 89·7 % out of a total average oxidation of 94·5 %.

Filter C_1 received 3 parts P_2O_5 per 100,000 as K_2HPO_4 and gave a daily average oxidation of sugar of 99·2 % over the first 43 days. It should be pointed out that differences of a few per cent. are of greater significance when the purification figure approaches 100 % than at, say, 50. The amount of work, measured by extra filter space, required to increase a percentage purification from 50 to 60 is much less than that required to produce the same increment from 90 to 100. Thus section 1 of C_1 oxidised 98 % of the total sugar but a small amount of sugar was still present in the final effluent.

In filter C_1 the growth of film covered the surface of the medium and by the end of the first month the solution dropping on to the filter did not pass through immediately but gathered in pools on the surface and drained through the filter at a slower rate than was desired. This condition of ponding led to the initiation of the second series of experiments, the results of which are described later.

Removal of phosphorus and nitrogen. When no phosphate was supplied the amount of sugar removed from solution was, as previously stated, small and the amount of nitrogen removed was also small. Out of a total of 5 parts N supplied as NH_4Cl an average of only 0·3 part was taken up over a period of 43 days (Table I). An addition of 0·3 part P_2O_5 to B_1 increased the average removal of N over the 43 days to 1·416 parts. The quantity of P_2O_5 added in this case apparently did not provide enough phosphorus to cause all the dissolved sugar to be oxidised since some sugar remained after all the phosphorus had been removed from solution. Not more than a trace of phosphorus was ever found in the final effluent from B_1. The figures obtained with filter C_1, however, showed that 3 parts P_2O_5 per 100,000 provided more than enough phosphorus. Practically all the sugar supplied was oxidised while only 1·63 parts of the P_2O_5 were removed. The fall in concentration of nitrogen in C_1 was 2·4 parts from an initial supply of 5 parts N.

Although it seemed unlikely that intermediate compounds such as hexosephosphate would be present in the effluents, as these substances do not ordinarily pass out of the living cell into the substrate, tests were made for the presence of soluble organic compounds of phosphorus in the effluents from the filters. The effluents were analysed for inorganic and organic forms of phosphorus as soon as sufficiently large volumes had been collected. In consequence the solutions were on an average 2 hours old at the time of analysis. The p_H values of the effluents of filters B_1 and C_1 were about 3·0. Under these conditions soluble organic compounds of phosphorus were invariably absent, although it is almost certain that at times organic phosphorus would be contained in the small amounts of film in suspension occasionally washed out of the filters. The effluents were settled for a few minutes so as to exclude visible suspended matter from the samples used for the determination of phosphorus.

In order to determine the amount of organic phosphorus which could be recovered in a typical hexosephosphate by the Parker and Fudge modification [1927] of the Denigès method, an examination was made of a sample of barium hexosephosphate, kindly supplied by Prof. A. Harden. The preparation contained 9·5 % moisture and the phosphorus content of the dry substance was approximately: total P_2O_5, 22·7 %; organic P_2O_5, 20·6 %; inorganic P_2O_5, 2·1 %. Solutions of 10 mg. of the barium compound per litre were made up so that there were present: total P_2O_5, 2·05 parts per million: organic, 1·87 parts; inorganic, 0·18 part. The corresponding figures as determined colorimetrically by the modified Denigès method were 2·1, 1·9, and 0·2 respectively, showing that it was reliable for the determination of the organic phosphorus in hexosephosphates. The p_H value of the distilled water in which the solutions were made up varied between 5·5 and 6·0. After storage for several hours it was observed that the amount of inorganic phosphorus in the hexosephosphate solutions had increased. Thus on one occasion 0·86 part inorganic P_2O_5 per million was found after the solution had stood for 8 hours, while on another occasion all the hexosephosphate had been completely hydrolysed after standing 24 hours at room temperature. It seems that very dilute aqueous solutions of hexosephosphate undergo hydrolysis on storage: the solutions were made up in distilled water but no precautions were taken to ensure that they were sterile. An alternative colorimetric method described by Zinzadzé [1932] was tried, since the colour standards are more permanent than in the Denigès method, but the hexosephosphate appeared to be hydrolysed to inorganic phosphorus during analysis, owing to the greater concentration of mineral acid used by Zinzadzé.

It has already been mentioned that the p_H values of the final effluents from filters B_1 and C_1 were about 3·0. When the source of nitrogen added was changed on the 68th day from ammonium chloride to ammonium bicarbonate, the p_H values of the solutions were either unchanged or increased on filtration through B_1 and C_1. The p_H values of the nutrient solutions supplied to the filters were 5·5 for B_1 and about 6·0 for C_1. Barritt [1931] filtered 0·2 % solutions of sucrose *plus* ammonium sulphate through sectional filters and found that the p_H of the liquid changed from an initial value of 7·0 to 5·5 in the second upper section and to 7·1 in the sixth and final section; the reduction and subsequent increase in p_H were attributed to the production and oxidation of organic acids. The following considerations indicate that the observed differences in the p_H values of the solutions supplied to the filter and of the final effluents in Exp. 1 (Table II) were due mainly to the removal of nitrogen from ammonium chloride and the liberation of an equivalent quantity of free HCl. This was not neutralised or absorbed by the glass medium and therefore passed out with the effluent. For example, on the 19th day of the experiment the amounts of nitrogen expressed in parts per 100,000 removed by filters A_1, B_1 and C_1 were 0·19, 1·09 and 1·83 respectively. Assuming that equivalent amounts of free HCl were liberated, the strength of acid in the effluents would be 0·137, 0·781 and 1·308 $N/1000$ for A_1, B_1 and C_1 respectively. Sufficient acid would thus be produced to cause the drop in p_H value from 5·6 in the nutrient solution to 5·0 in the final effluent of A_1, from 5·4 to 3·2 in B_1 and from 6·0 to 2·8 in C_1. If chlorine ions, equivalent to the nitrogen assimilated, were taken up by the filter this argument would be untenable. Determinations of Cl were made therefore with silver nitrate and the following results were obtained:

Table II. *Effect of source of nitrogen on formation of acid.*

Filters fed with sucrose solution and N as NH_4Cl (made up in distilled water) from start of experiment until the 68th day: after this they received N as NH_4HCO_3.

A_1 received no P_2O_5.
B_1 received 0·3 part P_2O_5 per 100,000.
C_1 received 3·0 parts P_2O_5 per 100,000.
N removed expressed as parts per 100,000.

Day of exp.	Nutrient solution	1st section		2nd section		3rd section		4th section		Filter
	p_H	p_H	N removed	p_H	N removed	p_H	N removed	p_H	N removed	
14	5·4	5·1	—	5·1	—	4·8	—	4·6	0·2	A_1
	5·8	3·6	—	3·2	—	2·9	—	2·9	1·47	B_1
	6·6	2·9	—	2·9	—	2·9	—	2·9	3·57	C_1
19	5·6	—	—	—	—	—	—	4·4	0·19	A_1
	5·4	—	—	—	—	—	—	3·2	1·09	B_1
	6·0	—	—	—	—	—	—	3·0	1·83	C_1
26	5·4	5·2	0·37	5·2	0·00	5·4	0·00	5·6	0·00	A_1
	5·0	3·0	1·59	3·0	0·08	3·0	0·00	3·0	0·34	B_1
	4·7	—	3·38	3·0	0·08	3·0	0·09	3·0	0·28	C_1
43	6·0	6·0	0·56	5·1	0·04	5·3	0·49	5·5	0·38	A_1
	5·6	3·0	1·54	3·2	0·00	3·2	0·00	3·3	0·89	B_1
	6·2	3·2	2·07	3·2	0·00	3·1	0·34	3·1	1·02	C_1
70	7·4	—	—	—	—	—	—	7·2	—	A_1
	7·3	—	—	—	—	—	—	6·9	—	B_1
	7·1	—	—	—	—	—	—	6·0	—	C_1
75	7·4	—	—	—	—	—	—	7·3	—	A_1
	7·3	—	—	—	—	—	—	6·8	—	B_1
	7·1	—	—	—	—	—	—	6·4	—	C_1

Cl in nutrient solutions and effluents from filters.

Parts per 100,000.

Filter	A_1	B_1	C_1
Cl in nutrient solution	14·29	14·29	14·29
„ effluent of section 1	14·26	14·26	14·87
„ „ 2	14·26	14·57	14·57
„ „ 3	14·27	14·72	14·72
„ „ 4	15·17	15·02	14·26

These results show that there was no uptake of Cl by the filters commensurate with the removal of nitrogen. If the decreases in p_H recorded with filters B_1 and C_1 were caused entirely by organic acids ionised to the same degree as acetic acid, then the concentration of acid required would be greater than 0·1 %. As the original amount of sugar was only 0·1 % a larger quantity of acetic acid could not be present. Moreover when determinations of the relative amounts of organic matter in the nutrient solutions and in the effluents were made by a test in which both sugar and organic acids are oxidised, *viz.* the biochemical oxygen demand, known as the "Dissolved oxygen absorbed test" in the Ministry of Health Methods [1929], results as follows were obtained:

Biochemical oxygen demand (B.O.D.) in 5 and 20 days.

Average results of five analyses made at different times on the liquids filtered and the effluents from the sections.

Parts per 100,000.

Filter	Crude liquor filtered		Effluent of section 1		Effluent of section 2		Effluent of section 3		Effluent of section 4	
	5 days	20 days	5 days	20 days	5 days	20 days	5 days	20 days	5 days	20 days
A_1	69·2	103·6	71·1	93·6	63·4	78·7	61·1	99·4	56·3	95·3
B_1	64·1	105·3	46·2	73·2	39·1	55·0	45·1	69·2	33·1	55·4
C_1	69·6	96·2	26·4	41·9	25·4	45·1	15·4	28·4	8·6	18·3

A 0·1 % solution of sugar, acetic or lactic acid has a maximum B.O.D. figure of about 112, which is approached in 20 days. Even if the whole of the B.O.D. were due to one of these organic acids there would still be insufficient acid to account for the observed fall in p_H value during filtration of the sugar solutions. This evidence indicates that the differences in p_H values of the original solution and filter effluents are caused by mineral acid set free from the ammonium salt, a conclusion not necessarily conflicting with that of Barritt, if the differences between the two experimental conditions are considered. The 0·2 % sugar which Barritt used may have given a growth of film in which acid production proceeded more quickly than oxidation of sugar and acid.

Experiment 2.

This experiment was undertaken because (*a*) two of the filters used in Exp. 1 showed signs of choking before the completion of the work, (*b*) the development of highly acid effluents consequent on the use of ammonium chloride as a source of nitrogen may have interfered with the development of organic film and nitrification processes. To provide a larger air space in the glass filters used for Exp. 2 the broken glass medium was replaced by tubes, 1 cm. diameter and 1·5 cm. long. The sectional filters employed were A_2, B_2 and C_2 and they received solutions of the same composition as were supplied to A_1, B_1 and C_1 respectively in Exp. 1, except that (1) nitrogen was supplied as ammonium bicarbonate, (2) the nutrient solution filtered in A_2 contained 0·05 g. K_2SO_4 per litre. A rate of flow of 2 litres per day, equivalent to 100 g.y.d., was maintained and the experiment was continued for 73 days.

Development of film. At the end of the experimental period of 73 days there was no film visible on any of the glass tubes in filter A_2; the filter did not appear to retain liquid as long as B_2 or C_2. The film in B_2 increased slowly in amount as the filter aged and apparently had not reached maximum activity at the termination of the experiment. Filter C_2 had a white gelatinous growth on the surface within a fortnight and after this the film spread downwards through the first and second sections. At the end of the experiment the first section had a thick, pale yellow, gelatinous film but it was not choked, and liquid could be filtered at twice the desired rate without surface ponding. Section 2 in filter C_2 contained much film, though this was less in amount than in section 1, while there was considerably less film in section 3 than in section 2. In section 4 the small quantity of film present consisted of a thin slime on the glass tubes. It was observed in the early stages of the experiment that much of the film in sections 2 and 3 was formed by growths in the section above being washed into the one below, forming centres of inoculation from which the film spread to other parts of the filter.

Table III. *Effect of supply of phosphate on sucrose oxidation and removal of nitrogen.*

Exp. 2. Glass filters and tubular glass medium: Rate of flow = 100 g.y.d.

Filter A_2 received sucrose equivalent to 105·3 parts glucose per 100,000 and no P_2O_5.
Filter B_2 received sucrose equivalent to 105·3 parts glucose per 100,000 *plus* 0·3 part P_2O_5.
Filter C_2 received sucrose equivalent to 105·3 parts glucose per 100,000 *plus* 3·0 parts P_2O_5.

2 litres of nutrient solution supplied daily = 100 g.y.d.

Parts per 100,000.

Day of exp.	P_2O_5 in final effluent		Sugar in final effluents: glucose equivalent			Nitrogen in final effluents (5 parts N supplied)		
	B_2 (0·3 supplied)	C_2 (3·0 supplied)	A_2	B_2	C_2	A_2	B_2	C_2
26	0	2·00	96·8	67·1	2·0	4·98	4·07	2·20
33	0	—	107·0	61·5	2·0	—	—	—
44	—	1·00	111·8	58·0	0·4	3·73	4·13	2·67
58	0	0·75	108·5	50·3	0·8	4·79	3·75	2·98
60	0	1·00	103·2	57·8	0·9	4·81	3·98	2·36
66*	0·002	2·60	96·3	33·7	1·5	5·07	4·47	3·70
71	—	—	106·4	58·4	7·1	4·36	3·75	3·51
73	0	1·38	90·0	34·0	4·6	4·94	3·68	1·87

* Filter C_2 received 4 litres daily from 61st day.

Oxidation of sugar (Table III). The amounts of sugar oxidised in the three filters A_2, B_2 and C_2 at different stages of the experiment are shown in Fig. 1.

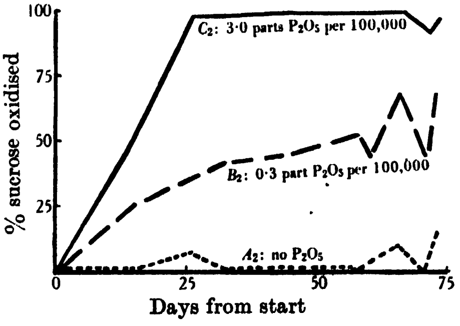

Fig. 1. Effect of phosphorus supplied on oxidation of sucrose

The curves indicate a definite relationship between the supply of phosphorus and the amounts of sugar oxidised, provided adequate additions of other essential mineral salts are made to the nutrient solutions. In the absence of added phosphorus filter A_2 rarely oxidised more than 14 % of the sugar, and the average amount oxidised over a period of 73 days was only 2·6 %. Filter B_2, however, with 0·3 part P_2O_5 per 100,000 decomposed 36 % of the carbohydrates after a period of 26 days. The percentage oxidation increased steadily to 50 after 50 days and on the 73rd day, at the end of the experiment, filter B_2 was oxidising 68 % of the sugar. The trend of the curve for B_2 suggests that the amount of sugar oxidised by this filter would have continued to increase if the experiment had been prolonged. The results show that the smallness of the amount of phosphorus supplied restricted the growth of a population sufficiently active to oxidise all the sugar. As the filter accumulated phosphorus and the film developed, the amount of organic matter oxidised increased. Two of the many factors which influence biological oxidation in percolating filters are thus seen to be the supply of phosphorus and the time factor. When the results obtained with filters B_1 and B_2 are compared a striking difference is observed in the amounts of sugar oxidised. After receiving 0·1 % solution of sugar daily for 43 days (Table I) filter B_1 oxidised 98 % daily whereas B_2 oxidised only 43 % after operating for the same period (Fig. 1). This comparison emphasises another important factor in filtration, *viz.* the relationship between the size of filtering medium and the efficiency of the filter. Apart from the change made in the source of nitrogen the only difference between B_1 and B_2 was that B_1 contained small pieces of solid glass while B_2 was filled with larger pieces of tubular glass. This difference in size of medium affects the phosphorus requirements of the filters in the following manner. Sufficient phosphorus must be provided to B_1 to build a film able to retain the solution passing through the filter long enough for the sugar to be decomposed. With the small grade of medium in B_1 a limited growth of organisms was sufficient to join up the particles of medium and so increase the time of contact of the filter. The quantity of phosphorus used for B_1, when supplied to B_2, gave about the same growth, but here the film only covered the surface of the glass tubes and did not join them together so as to make a continuous growth. Spaces were thus left in the filter through which sugar passed and escaped oxidation. With filter C_2, which received 3 parts P_2O_5 per 100,000, the amount of sugar decomposed reached 98 % in the first 26 days and later continued to improve until the oxidation attained was almost quantitative. Even when the rate of flow was doubled on the 60th day of the experiment over 93 % of the carbohydrate underwent oxidation.

The effect of supply of phosphorus on the contribution made by the different parts of the filter towards the total oxidation of the carbohydrate was found by dismantling the filters at various intervals and analysing the effluents of the different sections of the three filters. The results appear in Table IV. The samples were taken successively from the different sections over a period of 16 hours, beginning at section 4 and working upwards. Consequently the samples did not correspond exactly, and probably for this reason, the analytical results do not always show a regular decrease in sugar content from the inlet to the outlet of the filter. The figures in Table IV show that the effect of withholding phosphate from filter A_2 was to inhibit inversion and oxidation of sugar. Traces of invert sugar were generally present in the effluents from the first section of A_2 and in these cases the same quantity persisted almost unchanged in the effluents from lower sections. These small amounts of invert sugar may have been due to analytical errors in the Hagedorn-Jensen method as carried out by the author [Jenkins, 1933, 2]. With filter B_2 a high proportion of invert sugar always appeared in the effluent from section 1 with a tendency for the

Table IV. *Experiments with glass filters filled with tubular glass medium.*

Solution fed to filter A_2: Sucrose equal to 105·3 parts glucose; 5 parts N as NH_4HCO_3; no P_2O_5.

Solution fed to filter B_2: Sucrose equal to 105·3 parts glucose; 5 parts N as NH_4HCO_3; 0·3 part P_2O_5: per 100,000.

Solution fed to filter C_2: Sucrose equal to 105·3 parts glucose; 5 parts N as NH_4HCO_3; 3·0 parts P_2O_5: per 100,000.

Total sugar and invert sugar (given as glucose) in effluents from sections.

Parts per 100,000.

Day of exp.	Section 1		Section 2		Section 3		Section 4	
	Total sugar	Invert sugar	Total sugar	Invert sugar	Total sugar	Invert sugar	Total sugar	Invert sugar
	Filter A_2. 2 litres solution fed daily.							
15	93·2	3·1	90·4	3·0	88·8	2·8	88·2	2·8
26	103·5	0·5	101·5	0·8	100·3	0·5	96·8	0·4
30	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	107·0	0·7
44	110·3	0·5	111·8	0·5	111·8	0·8	111·8	0·8
58	—	—	—	—	—	—	108·5	1·2
60	101·0	1·0	107·2	1·0	103·2	1·0	—	—
66	—	—	—	—	—	—	96·3	1·5
71	—	—	—	—	—	—	106·4	3·3
73	—	—	—	—	—	—	90·0	1·7
	Filter B_2. 2 litres solution fed daily.							
15	81·2	17·1	63·5	16·2	69·7	15·8	65·0	14·8
26	59·2	29·9	89·3	35·4	78·0	36·4	67·1	30·7
30	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	61·5	56·4
44	74·0	25·2	80·0	30·1	65·0	37·3	58·0	50·1
58	—	—	—	—	—	—	50·3	26·0
60	49·0	23·4	58·0	24·8	48·7	27·9	57·8	33·7
66	—	—	—	—	—	—	33·7	24·0
71	—	—	—	—	—	—	58·4	26·6
73	—	—	—	—	—	—	34·0	25·3
	Filter C_2. 2 litres solution fed daily until 60th day; after that 4 litres fed daily.							
15	44·5	16·8	48·9	14·2	46·4	14·6	40·6	12·9
26	22·1	6·8	5·8	4·0	1·4	1·0	2·0	0·6
30	44·4	26·1	31·9	25·4	29·1	20·0	18·7	18·7
33	—	—	—	—	—	—	2·0	2·0
44	3·5	1·6	1·2	0·7	0·9	0·3	0·4	0·3
58	—	—	—	—	—	—	0·8	0·8
60	3·4	0·8	0·8	0·4	0·3	0·3	0·9	0·6
66	—	—	—	—	—	—	1·5	1·5
71	—	—	—	—	—	—	7·1	3·2
73	—	—	—	—	—	—	4·6	1·1

amount of invert sugar to increase on passage down the filter. On two occasions the sugar in the final effluent was largely the inverted product, there being 56·4 parts invert sugar in a total sugar content of 61·5 parts on the 33rd day and 50·1 out of a total of 58·0 on the 44th day. The view has previously been advanced [Jenkins, 1933, 2] that the occurrence of a large proportion of invert sugar in the effluents of a filter receiving solutions of sucrose is a sign of an immature film, and that increase in efficiency of oxidation brings about a decrease in the proportion of invert sugar in the total sugar. The observations made above on the composition of the effluents from the different sections support this view, since B_2 never reached maturity during the 73 days of the experiment. Although the film in B_2 was unable to oxidise the carbohydrates that passed through it, the filter was yet able to invert a large proportion of the sugar it received. The film in section 2 of filter B_2 was small in amount and considerably less in the two lower sections. All these sections however were able at times to bring about the inversion of appreciable quantities of sugar in the absence of measurable amounts of inorganic phosphate in the effluents (less than 0·002 P_2O_5 per 100,000) but were unable to carry the decomposition much beyond this stage. The addition of 3 parts P_2O_5 per 100,000 to the sugar solution fed to filter C_2 resulted in the growth of much film in section 1 and less in section 2. Only a very small amount of growth took place in sections 3 and 4, owing to the low concentration of sugar in the solutions reaching these sections. As previously observed, some of the film in section 2 was washed down from the upper part of the filter.

A large proportion of the sugar which passed through filter C_2 was hydrolysed to invert sugar: on the 30th day all the sugar contained in the effluent of section 4 appeared in this form. The concentration of sugar in effluent 4, filter C_2, was negligible after the 30th day as long as the rate of 100 g.y.d. was maintained.

Phosphorus and nitrogen removed. The quantities of phosphorus and nitrogen taken up by the filters are given in Table V. The figures show that with one exception the phosphate added to B_2 was removed quantitatively by this filter. Reference to Tables IV and V shows that section 1 of B_2 was responsible for the removal of the whole of the phosphate and for the oxidation of most of the sucrose. The absence of phosphorus from the solutions of sugar leaving this section accounts for the poor development of film in the remaining sections of this filter. Varying amounts of nitrogen were taken up by B_2 during the early stages of the experiment but later small amounts were removed continuously. An average of 1·02 parts N per 100,000 was removed from the solutions, which before filtration contained 5 parts. Table V shows that most of the nitrogen was absorbed by section 1 of B_2 and only small amounts by lower sections. The solutions leaving section 1 therefore contained sugar and ammonium salts but practically no phosphorus. On the other hand, filter C_2 received more than enough phosphorus for its requirements (Table V), an average of 1·54 parts P_2O_5 being taken from a supply of 3 parts. This uptake of phosphorus enabled C_2 to oxidise almost completely 2 litres of a solution of 0·1 % sucrose every day for the first 60 days and 4 litres daily after the 60th day of the experiment.

Table V. *Phosphate and nitrogen in effluents at different levels of the filters.*

Exp. 2. Glass filters and tubular glass medium: rate of flow - 100 g.y.d.

Filter A_2 received sucrose equivalent to 105·3 parts glucose per 100,000 and no P_2O_5.
Filter B_2 received sucrose equivalent to 105·3 parts glucose per 100,000 *plus* 0·3 part P_2O_5.
Filter C_2 received sucrose equivalent to 105·3 parts glucose per 100,000 *plus* 3·0 parts P_2O_5.

Parts per 100,000.

	26th day of Exp.				60th day of Exp.				
	P_2O_5		Nitrogen		P_2O_5		Nitrogen		
	B_2	C_2	B_2	C_2	B_2	C_2	A_2	B_2	C_2
Nutrient solution contained	0·3	3·00	5·00	5·00	0·30	3·00	5·00	5·00	5·00
Effluent of section 1 contained	Trace	2·00	4·04	2·23	0·00	1·00	4·92	4·36	3·40
,, 2 ,,	0	1·87	4·07	2·09	0·00	0·76	4·81	4·28	2·88
,, 3 ,,	0	1·50	4·07	1·92	0·00	0·88	4·81	4·17	2·77
,, 4 ,,	0	2·00	4·07	2·20	0·00	1·00	4·81	3·98	2·36

The average amounts of nitrogen retained by the filters are obtainable from the data given in Table V and show that the uptake of this element depends upon the quantity of phosphorus available for the growth of the film. Thus in the absence of phosphorus only 6·6 % of the N supplied to A_2 was removed: B_2 and C_2, which received 0·3 part and 3 parts P_2O_5 respectively, took up 20·5 and 44·9 % of N respectively.

Figures for the total amounts of C, N and P removed from solution by filters B_2 and C_2 during the experiment are presented in Table VI. The difference in the ratios of C, N and P in B_2 during the first 44 days and the last 29 days means that more carbon was oxidised for a given supply of N in the later stages of the experiment than in the earliest part. Although the C/N/P ratios for the last 29 days of B_2 and the whole period for C_2 are similar in respect of C and N yet the actual amounts of C and N oxidised and removed differed considerably in the two filters. The suggestion was made in a previous paper [Jenkins, 1933, 3] that nitrogen would be used most economically in a filter oxidising sugar if the initial C/N ratio were kept at about 30/1. A C/N ratio of 19·8/1 was obtained with C_2 because an excess of N was supplied. In the presence of less N the same amount of sugar would probably have been oxidised and C and N removed in a wider ratio. According to the figures given above, the minimum proportion of phosphorus required for the efficient utilisation of nitrogen would lie between 0·12 and 0·31 part available P (0·28 and 0·71 parts respectively P_2O_5) per part of nitrogen.

Table VI. *Carbon, nitrogen and phosphorus removed from solutions.*

C in sugar solutions supplied to B_2 and C_2 = 42·1.
N in sugar solutions supplied to B_2 and C_2 = 5·0.
P in sugar solutions supplied to B_2 and C_2 = 0·13.

Parts per 100,000.

	Filter B_2 Experimental period						Filter C_2 Experimental period		
	First 44 days			Last 29 days			73 days		
	C	N	P	C	N	P	C	N	P
	17·2	1·83	0·13	23·4	1·07	0·13	41·1	2·08	0·64
Ratio C/N/P	9·4/1/0·071			21·9/1/0·12			19·8/1/0·31		

As previously mentioned, the formation of acid in percolating filters was found to arise from the use of NH_4Cl as the source of nitrogen. In Exp. 2 slight variations were found in the p_H values of the solutions undergoing filtration (Table VII) but reductions in p_H of the order observed when ammonium chloride was the source of N were not detected. There is thus no evidence that intermediate organic acids were formed to any considerable extent during the aerobic oxidation of 0·1 % sugar by the mixed flora of the percolating filter operated under the conditions observed in these experiments.

Table VII. *Average* p_H *values of nutrient solutions and effluent.*

		Section effluents			
Filter	Nutrient	1	2	3	4
A_2	7·8	7·8	7·8	7·9	7·8
B_2	7·3	7·2	7·3	7·3	7·1
C_2	7·1	7·6	7·2	7·3	7·0

Experiments 3 (a) and 3 (b).

The availability of compounds of phosphorus in percolating filters.

Exp. 3 (a). The apparatus used for this experiment was the large scale filtration plant previously described by Richards and Cutler [1933]. The liquid treated by the biological filters was the effluent from the beet-pulp presses of a beet-sugar factory, diluted and partly fermented, so that it gave approximately the following results on analysis:

	Parts per 100,000
Oxygen absorbed in 4 hours from permanganate ...	122
Biochemical oxygen demand in 5 days	109
Biochemical oxygen demand in 20 days	156
Sucrose	68
Acetic acid	20
Lactic acid	20
Solids in suspension (4 % ash)...	42
P_2O_5, all organic	0·85
Nitrogen in organic combination	2·5
Nitrogen as ammonia	0·01

p_H, 5·1

The suspended matter consisted of cellulose and fibre derived from the sugar-beet root, while the organic nitrogen was mostly of protein origin and less readily available to micro-organisms than ammonia. The organic compounds of phosphorus were probably hexosephosphate, nucleoproteins and phospholipins derived from organisms and the cell contents of the beet. Liquid of the average composition given above was distributed at a rate of 50 g.y.d. over two filters. As the chief object of the experiment was to test the effect of inoculation on the rate of oxidation of the constituents of fermented press water, one of the filters was inoculated with organisms selected by Mr D. W. Cutler and Miss L. C. Crump, while the other filter was not inoculated. Despite this difference the final effluents of both filters were almost identical in chemical composition. Hence they have been considered as duplicates for the purpose of this experiment: the figures given are the averages of the analytical data from the inoculated and uninoculated filters. During the first 20 days the organic compounds of nitrogen and phosphorus present in the liquids filtered varied according to the composition of the factory press water, but known quantities of ammonia or potassium phosphate were added. The liquids filtered during this period could be classified as follows:

(1) For the first 7 days the liquids contained nitrogen as ammonia and in organic combination as well as 0·85 part P_2O_5 per 100,000 as organic phosphorus supplied by the pulp liquor.

(2) From the 8th day to 13th day the liquids contained nitrogen as ammonia and in organic combination besides phosphorus in organic combination and as added K_2HPO_4.

(3) From the 14th day to 18th day the substances in (2) were present, though the amounts of N and inorganic phosphorus were less.

(4) After the 20th day the liquids contained organic nitrogen, phosphorus in the organic and inorganic states and traces of ammonia.

The results of operating the filters under the conditions given above are summarised in Table VIII. The figures for percentage purification represent the averages of the amounts of oxidation of organic matter in the two filters as measured by the B.O.D. test. It must be pointed out that the conclusions

Table VIII. *Results of large-scale experiments.*

Parts per 100,000.

		P_2O_5 in liquid filtered		P_2O_5 recovered in effluent.	N in liquid filtered		N recovered in effluent		Oxidation of organic matter
Day of Exp.	Rate g.y.d.	Inorganic	Organic. Average figure	Inorganic	Ammonia N	Organic N	Ammonia N	Organic N	%
0–7	50 for 10 hrs. daily	0	0·85	0	3·00	1·85	2·50	1·12	25
8		3·0	,,	0·30	3·00	1·85*	2·00	—	28
10		,,	,,	0·50	3·00*	2·48*	(Total N = 1·49)		55
11		,,	,,	0·50	3·00*	2·38*	(Total N = 1·03)		81
12		,,	,,	0·41	3·00*	1·60*	(Total N = 0·78)		75
13		,,	,,	0·56	2·5	3·76	0·07	1·16	86
14	50	1·5	,,	0·38	1·5	3·34	0·05	1·49	86
15	50	,,	,,	0·02	2·25	2·00	0·03	1·21	85
16	50	,,	,,	0·02	1·00	2·16	0·01	1·25	78
18	100	,,	,,	0·22	—	—	—	—	66
20	100	,,	,,	0·75	0·08	2·80	0·03	—	51

* Approximate.

drawn should be regarded as tentative or as supporting those already given in the early part of the paper, for the reason that liquors of different composition were treated in the same filter in its early stages and not simultaneously in different filters. Hence comparisons are available only between the results of filtering liquids of different composition at different periods in a filter undergoing development.

If the results given above are considered with respect to the changes made in operating the filters the following conclusions may be drawn:

(1) *1st–7th days.* In the presence of organic phosphorus and nitrogen in organic and inorganic combination not more than 25 % of the organic matter was oxidised on filtration and only 17 and 39 % respectively of the inorganic and organic nitrogen supplied were removed by the filters. The amount of organic phosphorus removed from the liquid was not determined, but as an approximation it is assumed that the quantity was proportional to the uptake of organic nitrogen.

(2) *8th–13th days.* The response to the addition of an inorganic source of phosphorus on the 8th and on successive days was immediate. Inorganic phosphorus to the extent of 90 % was removed in the first 2 days and an average retention of 85 % was recorded over the period 8th to 13th days. The removal of inorganic nitrogen by the filters increased progressively from 33 % on the 8th day to 98 % on the 13th day, while the percentage purification increased from 28 to 86 in this period. This increase in percentage purification probably included the improvement in efficiency which generally accompanies the ageing or maturing of a filter. Cutler and Crump (unpublished work) found that the bulk of film rapidly increased in both filters between the 8th and 13th days.

(3) *14th–18th days.* A reduction in the amount of added phosphorus resulted in a greater percentage retention of P_2O_5, which increased to about 98. As long as ammonia was present, nitrogen in this form was removed almost quantitatively. The average oxidation of organic matter amounted to about 80 %.

(4) *20th day* et seq. When ammoniacal nitrogen was withheld the removal of inorganic phosphorus decreased and a rapid fall in the percentage oxidation followed. In the continued absence of inorganic nitrogen the percentage purification of organic matter remained at a low figure, in spite of the presence of an inorganic compound of phosphorus.

The figures above therefore show that (*a*) the phosphorus in the organic compounds contained in the press water of a sugar-beet factory is not so readily available to the micro-organisms in a percolating filter, even in the presence of sufficient available nitrogen, as an inorganic supply of phosphorus such as K_2HPO_4; (*b*) in the presence of potassium phosphate, nitrogen as ammonia is removed by the micro-organisms of a filter in preference to the organic compounds of nitrogen present in press water, with consequent improvement in the percentage purification of organic matter by the filter; (*c*) the removal of phosphorus as dissolved phosphate by a filter is slow and incomplete if the liquids filtered do not contain nitrogen in a form which can be rapidly utilised by the micro-organisms responsible for the activity of the filter. Press water appears to be deficient in this form of nitrogen.

Exp. 3 (*b*). The last conclusion, (*c*), received substantial corroboration from the results of an experiment briefly described below.

Two small laboratory filters were filled with gravel and supplied with solutions of the same composition as those used for Exp. 3 (*a*), at a rate of 100 g.y.d. Sufficient K_2HPO_4 was added to the solution fed to one filter to provide 1·5 parts P_2O_5 per 100,000: no such addition was made to the liquid supplied to the control filter. The crude liquor of both filters contained approximately 0·85 part P_2O_5 as organic phosphorus during the experimental period of 26 days. Analyses were made on 21 days and the following average results for percentage purification were obtained:

Average percentage purification of diluted press water.

Control filter received press water containing 0·85 part organic P_2O_5 per 100,000.

Phosphate filter received press water containing 0·85 part organic P_2O_5 and 1·5 parts inorganic P_2O_5 per 100,000 added as K_2HPO_4.

Oxygen absorbed from $N/8$ $KMnO_4$ % purification		B.O.D. in 5 days % purification		B.O.D. in 20 days % purification		% removal of total sugar	
Control	Phosphate	Control	Phosphate	Control	Phosphate	Control	Phosphate
46	52	44	38	36	37	53	37

In any one of the above tests the differences between the averages of the percentage purifications for the control and the phosphate filter are not significant.

The experiment therefore shows the oxidation of the organic matter in fermented press water by a percolating filter to be unaided by the addition of an available supply of phosphorus, if only those organic compounds of nitrogen normally found in press water are present.

DISCUSSION OF RESULTS.

The experiments described in this paper demonstrate that the influence of phosphorus on the rate of oxidation by a percolating filter of carbohydrates and the organic matter present in the press water of a beet-sugar factory is dependent on several factors, *viz.* (*a*) size of grade of filtering material, (*b*) the amount and nature of the compounds of phosphorus present, (*c*) the nature of the compounds of nitrogen present.

In order to build up a film able to oxidise a given quantity of carbohydrate at a given rate of flow a filter filled with $\frac{1}{16}$ to $\frac{1}{4}$ in. medium requires a smaller amount of available phosphorus than a filter with a coarse grade of material such as glass tubes 1·5 cm. long and 1 cm. bore. The primary function of the phosphorus is to supply the nutrient necessary for the growth of enough film to increase the time of passage of the dissolved organic matter through the filter. The results of Exps. 1 and 2 bring out the difference caused by filtering solutions of the same composition through filters filled with different grades of material. Filter B_1, with a small size of medium, received 0·3 part P_2O_5 per 100,000 and decomposed daily 98 % of a 0·1 % solution of sucrose after the filter had been in operation for one month, while B_2, which contained a larger grade of medium, but received the same amounts of phosphorus and sugar, oxidised less than 50 % of the sugar after operating for the same period. Filters with too fine a grade of medium suffer from the disadvantage of choking whenever vigorous growth of film occurs. Thus, increasing the supply of phosphorus to 3 parts P_2O_5 per 100,000 in Exps. 1 and 2 led to the choking of the fine medium in filter C_1, whereas the coarse medium in filter C_2 showed no signs of ponding although it received solution of the same composition as that passed through C_1. This observation might be of importance in actual practice where the ponding of filters increases operating difficulties and lowers the efficiency of the filter.

When 0·1 % solutions of sucrose containing 0·3 part P_2O_5 per 100,000 were filtered the phosphorus was, with one exception, quantitatively absorbed by the filter. An increase to 3 parts P_2O_5 in the liquids undergoing filtration led to a removal of about 1·5 parts P_2O_5 and the remainder passed out as inorganic phosphate with the effluent. Organic compounds of phosphorus were never detected in the effluents from any sections of the filters, although by the method used [Parker and Fudge, 1927] it was possible to determine as little as 0·002 part P_2O_5 as hexosephosphate per 100,000 of solution. Barritt [1931] treated a 0·2 % solution of sucrose, containing about 23 parts P_2O_5 per 100,000 as mixed potassium phosphates, in a percolating filter and found that the whole of the phosphate was not taken up by the filter. Even when one-sixth of this quantity of phosphate was used, *viz.* about 4·0 parts P_2O_5 per 100,000, he observed that inorganic compounds of phosphorus still appeared in the effluents. The results given in this paper would suggest that the occurrence of phosphate in the effluents from Barritt's filters was the result of supplying the filter with more phosphate than it could remove from solution. No evidence was found to indicate that hexosephosphate appeared in the effluents and became hydrolysed during analysis. Harden [1923] supposes that hexosephosphates are formed within the living cell first by the diffusion of sugar from the substrate through the cell wall, followed by the union of sugar with the phosphate already present in the cell. This compound is then split up into simple products and the phosphate which is regenerated is again able to unite with sugar.

The experiments on the filtration of press water from a beet-sugar factory show that hitherto unsuspected differences may exist as regards the availability of compounds of phosphorus in a percolating filter. In previous work reported by Richards and Cutler [1933] an ample supply of available nitrogen was recognised as essential for the rapid oxidation of dissolved organic impurities. The organic compounds of nitrogen and phosphate in press water probably have varying degrees of availability. Such compounds are removed mechanically by the filter before being assimilated by micro-organisms. The rate of oxidation of the organic matter passing through the filter is limited by the rate at which the nitrogen becomes available and this rate does not seem to be increased by supplying compounds of phosphorus which are readily utilisable by micro-organisms. If, however, nitrogen exists in the filter in an easily assimilable form such as ammonia, the rate of oxidation of organic matter is considerably increased by the supply of an available form of phosphorus.

Solutions of sugar and ammonium chloride suffered a considerable reduction in p_H on filtration, from a figure of 5·4 in the nutrient solution to 3·2 in one effluent, and from 6·0 to 2·8 in another. As previously mentioned, tests for the amount of dissolved organic matter showed that the decrease in p_H was too great to be accounted for solely by the production of organic acids from the sugar: negative results were obtained on testing for simple organic acids in the final effluents. It was also observed that the concentration of chloride in the solution undergoing filtration remained practically constant throughout its passage down the filter. Furthermore the p_H of the nutrient solution was not reduced appreciably when ammonium bicarbonate was substituted for ammonium chloride. These facts all indicate that the acid production in a percolating filter operated under the conditions described herein is chiefly derived from the mineral acid liberated by the removal of ammonium ion from the chloride or sulphate. Barritt [1931] found that organic acids may be produced in the upper part of a percolating filter if 0·2 % solutions of sugar are filtered: a reduction in p_H is observed in that part of the filter but the acid is oxidised as the solutions pass downwards, with corresponding increases in the p_H values of the effluents.

SUMMARY.

1. Experiments have been carried out on the biological oxidation in percolating filters of (*a*) 0·1 % sucrose *plus* different amounts of phosphate as K_2HPO_4 and with NH_4Cl or NH_4HCO_3 as the source of nitrogen, using broken glass as the filtering medium; (*b*) 0·1 % sucrose *plus* NH_4HCO_3 and different amounts of phosphate, with glass tubes as the filtering medium; (*c*) diluted press water from a beet-sugar factory; in this case the effect of organic componds of phosphorus on the oxidation of organic matter was studied in the presence of sources of nitrogen readily available to micro-organisms, and in the presence of compounds of nitrogen available only with difficulty.

2. The amount of phosphate required to ensure rapid oxidation of sugar in a 0·1 % solution is influenced to a large extent by the nature of the filtering medium. To ensure freedom from choking, the medium should be of a large size and of a suitable shape. Under these conditions a 0·1 % solution of sugar requires at first a maximum of about 1·5 parts P_2O_5 and 2·2 parts N per 100,000 for complete oxidation of the sugar; once the film becomes mature 0·3 of P_2O_5 and 1 of N.

3. Excess of phosphate supplied to the filters passes out apparently unchanged. No organic compounds of phosphorus were ever found in the effluents from any of the sections of the different filters.

4. The organic compounds of phosphorus in the press water of a beet-sugar factory are only slowly available to the micro-organisms on a percolating filter as compared with potassium hydrogen phosphate, and this may limit the rate of oxidation of organic matter by a percolating filter. In the absence of a form of nitrogen which is readily assimilated by micro-organisms the full effect of a supply of available phosphorus is not shown.

5. When percolating filters filled with glass medium were supplied with sugar, ammonium chloride and various amounts of K_2HPO_4 the p_H values of the solutions before and after filtration were:

(*a*) 5·6 and 5·0 respectively, without P_2O_5;
(*b*) 5·4 and 3·2 respectively, with 0·3 part P_2O_5 per 100,000;
(*c*) 5·0 and 2·8 respectively, with 3·0 parts P_2O_5 per 100,000.

The cause of acid production was found to be the removal of NH_3 from NH_4Cl and the liberation of free HCl. Quantitative tests and measurements of the dissolved organic matter present in the effluents showed that this acidity was not due to organic acids.

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REFERENCES.

Barritt (1931). *Biochem. J.* **25**, 1419.
Harden (1923). Alcoholic fermentation, p. 143. (Longmans, Green and Co., London.)
Jenkins (1933, 1). *Biochem. J.* **27**, 240.
—— (1933, 2). *Biochem. J.* **27**, 245.
—— (1933, 3). *Biochem. J.* **27**, 258.
Ministry of Health (1929). Methods of chemical analysis as applied to sewage and sewage effluents. (H.M. Stationery Office, London.)
Parker and Fudge (1927). *Soil Sci.* **24**, 109.
Richards and Cutler (1933). The purification of waste waters from beet-sugar factories. Water pollution research, technical paper No. 3. (H.M. Stationery Office, London.)
Zinzadzé (1932). *Z. Pflanz. Dung. Boden.* **23** A, 447.

XVII. A STUDY OF SEASONAL VARIATION IN BUTTER FAT.

II. A SEASONAL SPECTROSCOPIC VARIATION IN THE FATTY ACID FRACTION.

BY ROLAND GORDON BOOTH AND STANISŁAW KAZIMIERZ KON
National Institute for Research in Dairying, University of Reading,
AND WILLIAM JOHN DANN AND THOMAS MOORE
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council.

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IN previous communications [Booth *et al.*, 1933, 1, 2], the seasonal variations in the carotene and vitamin A contents of the butter fat produced from typical English milk were described, and a seasonal variation in the power of butter fat to inhibit the reaction between vitamin A and antimony trichloride was established. We also mentioned that the fatty acids prepared from summer and winter butter fats appeared to show certain differences, namely (1) acids from summer butter fats showed more intense absorption at $230 m\mu$ than those from winter butter fats, and (2) summer acids turned brown more rapidly than winter acids when treated with the antimony trichloride reagent. It was not clear at the time whether or not these two properties were related to the greater inhibitory power of summer butter fat in the antimony trichloride test. Further work has now demonstrated that neither the $230 m\mu$ absorption nor the rate of brown colour formation can be correlated with the inhibitory power. The rate of brown colour formation, indeed, is greatly increased in aged or oxidised fats, and any decision as to whether a seasonal variation of this property does actually exist must be postponed until one is in a better position to understand the nature of the reaction involved. On the other hand we have satisfied ourselves that the variation in absorption at $230 m\mu$ is of a genuinely seasonal character. Data in support of this view are given below.

EXPERIMENTAL.

Collection of butter fat etc. 15 specimens of butter fat were examined, selected from a large number of samples accumulated between April 1932 and May 1933. The period investigated thus included two occasions on which the cows were turned out to grass after stall feeding during the winter. The fatty acids were prepared by the following routine method: 5 g. of the butter fat were boiled for 2 minutes with 2·2 ml. of saturated aqueous KOH and 10 ml. of alcohol. The mixture was then diluted with 40 ml. of water and extracted twice with 75 ml. of ether each time, in order to remove the unsaponifiable matter. 20 ml. of 10 % sulphuric acid were then added and the free fatty acids extracted with 75 ml. of ether. The ethereal solution was washed twice with 10 ml. of water each time (with vigorous shaking) and once with 50 ml. of water (with gentle agitation) and dried by filtering through anhydrous sodium sulphate. The ether was evaporated under partial vacuum, and the residual fatty acids were transferred to a specimen tube.

Spectroscopic examination of the fatty acids. For the spectroscopic examination a Hilger E 3 quartz spectrograph with echelon cell photometer was used throughout. Solutions of the different samples of fatty acid were made in pure cyclohexane for the spectroscopic examination. A suitable concentration was found to be about 25 mg. in 10 ml. of the solvent.

Seasonal variation of intensity of absorption by the fatty acids at 230 $m\mu$.

The variation in the intensity of absorption at 230 $m\mu$ is shown in Fig. 1 (continuous curve). It will be seen that for winter butters $E^{1\%}_{1\,cm}$ for the acids has a value of 10 or slightly less (minimum value observed April 19–20, 1933 = 8·4) whereas the value for acids from summer butters is increased to 20 or more (maximum value observed, May 12–13, 1932 = 25·0)[1]. The increase in absorption, as judged from data obtained in the springs of 1932 and 1933, takes place coincidently with the cows obtaining access to grass. In both seasons significant increases were observed after one week of pasture feeding.

The effect of prolonged saponification on the absorption of the butter fat acids.

In our experiments the only departure from the standard method of saponification occurred during the early stages of the work, when a few of the specimens were saponified for the unnecessarily long period of 1 hour, instead of the usual period of 2 minutes. To rule out any possibility of a simulation of a seasonal variation through differences in the duration of saponification, acids prepared from typical winter and summer butter fats were submitted to prolonged boiling under reflux (15 hours) in alcoholic potash. A slight increase in the absorption

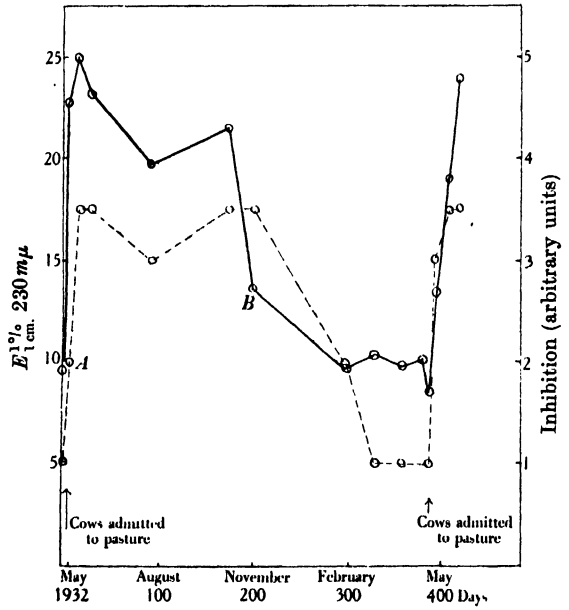

Fig. 1. --- Inhibitory power of butter fat. —— Absorptive power of acids of butter fat.

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[1] Slightly higher figures were obtained in subsequent determinations outside the series from which Fig. 1 was drawn.

of the winter butter acids was observed. It was, however, quite inadequate to account for the disparity between the absorption of typical winter and summer acids. A slight increase, probably falling within the range of experimental error, was also observed in the absorption of the summer acids.

	1 min. sap.	15 hrs. sap.
Winter acids, Feb. 24–27, 1933	12·6	15
Summer acids, May 19–22, 1933	27·1	30·0

Differentiation of 230 mμ *absorption and inhibitory power in $SbCl_3$ reaction.* In Fig. 1 the inhibitory power of the butter fat is indicated by the broken curve and the absorption of the acids by the continuous curve. Except for a few samples (notably those of May 3–4, 1932, marked *A* in Fig. 1, and Oct. 31, Nov. 3, 1932 marked *B*) there is a fair degree of parallelism between 230 *m*μ absorption and inhibitory power. To test this correlation samples of a typical summer butter fat (May 19–22, 1933) were submitted to various treatments and the effects on absorption and inhibitory power compared. It was found that whereas ageing and irradiation of the fat led to a marked decrease in inhibitory power, the absorption at 230 *m*μ was only slightly decreased. Aeration of the hot fat on the other hand caused reductions both in absorption and inhibitory power. To indicate the degree of severity of the various treatments iodine value determinations were carried out.

Table I. *Inhibitory power of butter fat.*

Butter fat of May 19–22, 1933. The inhibitory power has been expressed in arbitrary units by noting the depression in the blue reading caused by adding 0·5 ml. of the fat to 8 γ of B.D.H. vitamin A concentrate before the addition of 2 ml. of $SbCl_3$ reagent. The blue reading was taken immediately after adding the reagent. In the case of the aged, irradiated and oxidised specimens brown colorations developed on standing much more rapidly than with the untreated fat. Iodine values were determined by the Wijs method.

Treatment	Details	$E^{1\%}_{1\,cm}$ 230 *m*μ	Blue reading in absence of fat	Blue reading in presence of fat	Inhibitory power	I.V.
Untreated	—	25·9	7	2	5	48·5
Ageing	Fat was kept for several weeks in shallow layer in bottle on laboratory bench	18·8	7	5	2	46·9
Irradiation	Fat was exposed for 20 mins. to irradiation by mercury arc lamp at distance of 20 cm.	21·5	7	5	2	46·5
Oxidation	Fat was melted on water-bath and aerated by passing a current of air for about 24 hrs.	11·0	7	5	2	43·4

DISCUSSION.

230 mμ *absorption.* The fact that the mixed fatty acids of butter fat show an absorption maximum at 230 *m*μ was first pointed out by Gillam *et al.* [1931]. The band, however, is by no means specific and is observed in varying intensity in most fatty acids, *e.g.* in the mixed fatty acids of cod-liver oil [Edisbury *et al.*, 1933]. The main interest of the present work lies in the fact that the magnitude of the seasonal increase in absorption cannot be accounted for on the basis of our accepted knowledge of the relative composition of winter and summer butter fats and of the extinction coefficients of their known constituents. Since Ramart-Lucas *et al.* [1930] have shown that all saturated acids have very small and similar extinction coefficients at 230 $m\mu$, our attention, in attempting to account for the absorption of the mixed butter acids, may be restricted to oleic and linoleic acids, which according to Hilditch and Sleightholme [1930] are the main unsaturated components. In Table II the percentage of acids present has been taken from data obtained by Hilditch and Sleightholme with butters similar to those examined in the present work. The extinction coefficients for oleic and linoleic acids were observed by Dann and Moore [1933], using commercial preparations of acids, and may be taken as maximum values which probably would exceed those shown by the pure freshly prepared acids.

Table II.

	Absorbing component	% present in mixed acids	$E_{1\,\mathrm{cm}}^{1\%}$ 230 $m\mu$	Contribution towards E of mixed acids
Winter butter acid (coconut cake feeding)	Oleic	31·7	16·5	5·23
	Linoleic	2·4	9·6	0·23
		Total calculated E		5·46
		Observed		8·4
		Excess of observed over calculated		54 %
Early summer butter acids	Oleic	40·9	16·5	6·75
	Linoleic	4·1	9·6	0·39
		Total calculated E		7·14
		Observed		25·0
		Excess of observed over calculated		250 %

Two explanations for the excess of the observed over the calculated absorption seem possible. (1) Small amounts of unknown acids having very high extinction coefficients may be present in butter fat, the concentration being relatively low in winter and considerably increased in summer. (2) One or more of the known acids may be present in butter fat in a more highly absorptive form than that in which it exists in specimens prepared from vegetable sources. The second of these explanations is made attractive by our observation, reported in an accompanying communication [Dann *et al.*, 1935] that when certain oils are fed to the cow there is evidence of the appearance in the butter fat of acids having much higher extinction coefficients than the acids originally present in the oils. Although no definite decision can be reached as to the exact nature of the acids causing absorption, it seems probable that acids of the linoleic type, and possibly also more highly unsaturated acids, are involved.

Differentiation of 230 $m\mu$ *absorption and inhibitory power of the* $SbCl_3$ *reaction.* Although the above experiments permit no direct advance in our knowledge of the substances present in summer butter fat which cause inhibition of the blue coloration in the vitamin A test, it may be inferred that these substances, whatever their chemical character may prove to be, are extremely sensitive to oxidation and to irradiation. In these respects they are much less stable than the substances responsible for absorption at 230 $m\mu$, which are affected only by more vigorous treatment.

SUMMARY.

1. The absorption of the fatty acids of butter fat at 230 $m\mu$ shows a pronounced seasonal variation, being more than doubled during the summer months when the cows have access to pasture.

2. The increase in absorption runs approximately parallel with the increased inhibitory power of the butter fat in the antimony trichloride reaction for vitamin A as previously described. The substances responsible for inhibitory power, however, are much more sensitive to oxidation and irradiation than those responsible for 230$m\mu$ absorption.

3. The increased absorption of the acids from summer butter fat must be ascribed either to the presence of small amounts of unrecognised acids possessing high extinction coefficients or to the presence of modified forms of known unsaturated acids in a highly absorbing state.

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REFERENCES.

Booth, Dann, Kon and Moore (1933, 1). *Chem. Ind.* **52**, 270.
—— Kon, Dann and Moore (1933, 2). *Biochem. J.* **27**, 1189.
Dann and Moore (1933). *Biochem. J.* **27**, 1166.
—— —— Booth, Golding and Kon (1935). *Biochem. J.* **29**, 138.
Edisbury, Morton and Lovern (1933). *Biochem. J.* **27**, 1451.
Gillam, Heilbron, Hilditch and Morton (1931). *Biochem. J.* **25**, 30.
Hilditch and Sleightholme (1930). *Biochem. J.* **24**, 1098.
Ramart-Lucas, Biquard and Grunfeldt (1930). *Compt. Rend. Acad. Sci.* **190**, 1196.

XVIII. A NEW SPECTROSCOPIC PHENOMENON IN FATTY ACID METABOLISM. THE CONVERSION OF "PRO-ABSORPTIVE" TO "ABSORPTIVE" ACIDS IN THE COW.

BY WILLIAM JOHN DANN AND THOMAS MOORE
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

AND ROLAND GORDON BOOTH, JOHN GOLDING AND STANISŁAW KAZIMIERZ KON
National Institute for Research in Dairying, University of Reading.

(*Received November 7th, 1934.*)

IN an accompanying communication [Booth *et al.*, 1935] we have reported that when the cow is turned out to pasture after the winter the fatty acids obtained from the butter fat show greatly increased absorption for radiation of wave length $230 m\mu$. The work described in the present paper owes its origin to an attempt to correlate this increased absorption with the marked fall in the amount of fat in the milk which, as is already known, also frequently occurs when the cow first obtains access to green pasture. Now past work has shown that the feeding of cod-liver oil likewise causes a marked diminution in the amount of butter fat in the milk [Drummond *et al.*, 1924]. Further, cod-liver oil was reputed to yield acids having very high absorption for radiation of wave length $230 m\mu$ [Gillam *et al.*, 1931]. It seemed possible, therefore, that the depression of the fat might be linked up with the presence in it of highly absorbing[1] acids, derived either from grass or from cod-liver oil.

This explanation in its simplest form was ruled out when it was found by Dann and Moore [1933] that the high absorption of the fatty acids of cod-liver oil, recorded by Gillam *et al.*, is not observed if the acids are prepared by a brief saponification, but only after the acids have been submitted to prolonged treatment with the hot alkali. It remained possible, nevertheless, that acids which resembled those of cod-liver oil in showing increased absorption after suitable treatment might exert a similar depressing influence on the amount of fat in milk. Support for this view was afforded by early experiments in which we found that the absorption of the butter fat was indeed greatly increased when the relatively non-absorbing cod-liver oil was fed to the cows.

Further experiments have shown that fats capable of depressing the milk fat certainly do as a general rule possess unusual potency in raising the absorption at $230 m\mu$. The same property, however, is shared, if in a less degree, by other fats possessing no noticeable depressing action. The increase of absorption would indeed appear to be of a general character. It may occur in two ways, either *in*

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[1] The word "absorption" is used throughout the present communication to designate the extinction coefficient at the head of an absorption band at $230 m\mu$. Unless specifically excepted in the text, all figures for the extinction coefficient $E^{1\%}_{1\,\text{cm.}}$ at $230 m\mu$ (shortened for brevity to $E/230$) refer to the head of a band.

vitro as the result of chemical treatment, or *in vivo* in the course of the absorption of the fats by the cow and their secretion among the constituents of milk fat. The present communication is devoted to the description of this phenomenon. The possible bearing of our results on the nature of the substances responsible for the depression of the milk fat is being postponed for discussion elsewhere.

EXPERIMENTAL.

The conversion of "pro-absorptive" into "absorptive" acids by chemical means[1].

Experiments in vitro. Data relating to the absorptive properties and iodine values of the mixed acids obtained from the five oils used in our feeding experiments (two specimens of cod-liver oil, sardine oil, linseed oil, rape oil), both as prepared by a short saponification and after prolonged treatment of the acids by boiling with alcoholic potash, are given in Table I. The acids were prepared by the method described in the accompanying paper [Booth *et al.*, 1935]. Prolonged treatment with alkali was applied to the acids after their preliminary preparation and separation from unsaponifiable matter by the routine 2 min. saponification. Any complication arising through the possible action of the alkali on the unsaponifiable matter was thereby avoided.

It will be seen that for each oil the acids prepared by a short saponification showed only low absorption at $230 m\mu$. As the result of prolonged treatment with alkali there was in every instance at least a tenfold increase in absorption. In regard to the iodine values slight falls were observed in the acids from crude cod-liver oil (8 % decrease), medicinal cod-liver oil (10 %) and linseed oil (7 %). There was no change in the iodine values of acids from sardine oil and rape oil.

Table I. *Influence of prolonged treatment with alcoholic potash on the absorption of various fatty acids.*

Mixed acids from	Time of treatment	$E/230$	I.V.
Sardine oil (crude)	2 mins.	7·7	168
	48 hrs.	185	167
Cod-liver oil (crude)	2 mins.	7·9	166
	48 hrs.	196	153
Cod-liver oil (Engelberg's medicinal)	2 mins.	8·6	185
	37 hrs.	121	166
Linseed oil	2 mins.	6·3	188
	48 hrs.	67·9	181
Rape oil (deodorised)	2 mins.	5·0	114
	48 hrs.	65·8	115

The conversion of "pro-absorptive" acids into "absorptive" acids in vivo.

Feeding experiments. The feeding experiments extended over the late winter and early spring seasons of 1933 and 1934, the absorption of the butter acids on each occasion having reached its minimum level as a result of the fact that the cows had subsisted on stall feeding since the previous autumn. The oils, or oil fractions, were usually fed twice daily. The butters were prepared, the fats rendered and the fatty acids prepared according to routine methods[1].

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[1] A "pro-absorptive" fat or fatty acid is one which itself possesses little specific absorption but is capable of being converted into one which possesses such absorption, either *in vitro* or *in vivo*.

Experiments of Series I (1933). *The effect of feeding crude cod-liver oil and crude sardine oil on the absorptive properties of the mixed butter acids.* Two cows (Lena 3 and Welcome 7) were given 2 ozs. of crude cod-liver oil twice daily on March 14th, 3 ozs. on March 17th and 4 ozs. on March 20th, from which date 8 ozs. per day was continued in two doses till the end of the experiment. Two other (Flora 6 and Winsome 2) were given similar doses of crude sardine oil. The butter fats of Lena 3 and Welcome 7 were compounded, but since Winsome 2 took her sardine oil incompletely her butter was not compounded with that of Flora 6. By an oversight no samples of butter were taken from any of these cows to establish that the absorption of the butter acids had actually reached the typical winter level before the commencement of dosing. The results obtained during oil feeding may be compared however with those obtained upon butter given by the same cows after the cessation of dosing, or upon the butter given by the typical herd receiving the basal ration only during the period of dosing. The results obtained are given in Table II. It will be seen that as the result of feeding either cod-liver oil or sardine oil the absorption of the butter acids was greatly increased

Table II. *Exp. 1. Crude cod-liver oil and crude sardine oil feeding.*

Cows	Date of collection of milk	Remarks	$E/230$ of mixed acids
Typical herd	March 31–April 4	No oil given	13·1
Lena 3 and Welcome 7	March 30–April 3	4 ozs. cod-liver oil twice daily	48·4
Lena 3 and Welcome 7	April 20	After discontinuation of cod-liver oil feeding	18·0
Flora 6	March 30–April 3	4 ozs. sardine oil twice daily	42·8
Winsome 2	March 30–April 3	4 ozs. sardine oil twice daily, badly consumed	28·6
Flora 6 and Winsome 2	April 20	After discontinuation of sardine oil feeding	12·0

Experiments of Series II (1934). *Feeding experiments with linseed oil and rape oil.* Two cows (Flora 16 and Flora 20) were first given twice daily 2 ozs. of deodorised rape oil on February 19th, on March 6th this was increased to 3 ozs. and on March 9th to 4 ozs. On March 14th the rape oil feeding was discontinued and 3 ozs. of linseed oil were given twice daily to each cow. This was increased to 4 ozs. on March 16th. Since the sample taken during the feeding of linseed oil was prepared from milk collected 13 days after the discontinuation of the rape oil, there would appear to be little danger of the previous diet seriously affecting the result obtained. In this experiment also control samples were unfortunately not taken before the commencement of dosing, but the average value for specimens collected daily between April 7th and April 12th (about 1 week after the discontinuation of linseed oil feeding) may be accepted as a normal control.

The value $E/230 = 14{\cdot}4$ obtained for the butter acids during the rape oil period was only slightly higher than the value observed for acids obtained from the same cows after the discontinuation of dosing, and it is doubtful whether it was significantly higher than values around 10 which we would consider typical for winter butter fat. The value (20·0) obtained for the acids during the feeding of linseed oil showed a definite increase well beyond the range of experimental error.

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[1] Since our experimentswere not all carried out with the same immediate object in view it is unavoidable that there should be some lack of uniformity in the collection and presentation of results. Thus in some experiments the effect of feeding oil has been examined only by taking single specimens of butter fat at arbitrary periods after the commencement of oil feeding, in other experiments specimens have been collected daily over considerable periods.

Table III.

Cows	Inclusive dates of collection of milk	Remarks	*E*/230 of mixed acids
Flora 16 and Flora 20	p.m. March 13–a.m. March 14	4 ozs. of rape oil twice daily	14·4
Flora 16 and Flora 20	p.m. March 27–p.m. March 28	4 ozs. of linseed oil twice daily	20·0
Flora 16 and Flora 20	April 7–April 12	Average value over the period mentioned. Linseed oil feeding discontinued on March 30, p.m.	11·8

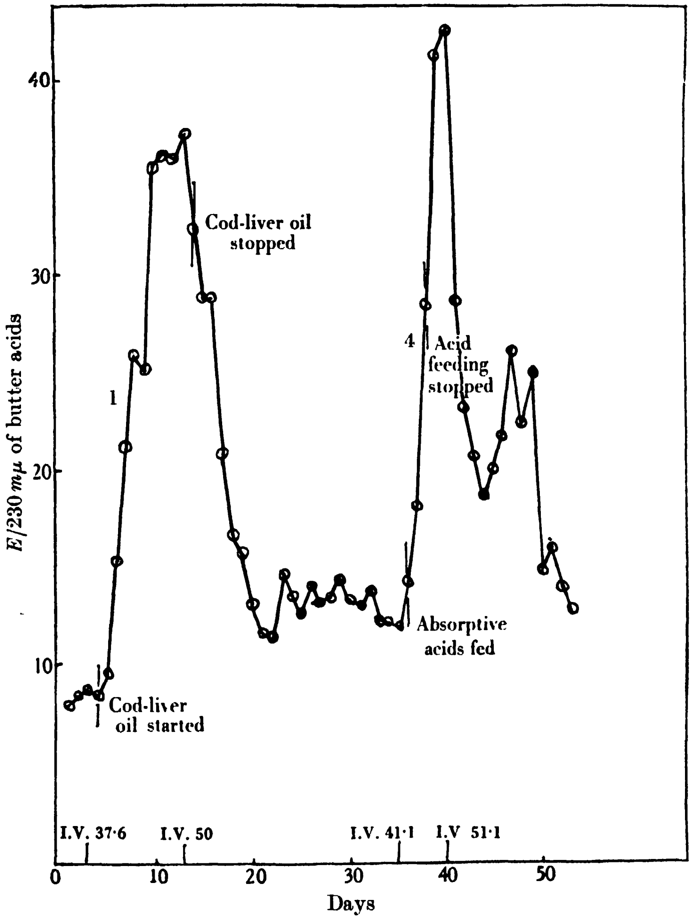

Fig. 1. Welcome 7. (1) Cod-liver oil feeding; (4) absorptive acid feeding.

Experiments of Series III (1934). *The feeding of medicinal cod-liver oil* (*Engelberg's*). In this series of experiments specimens of the butter acids were prepared daily. It has therefore been possible to study the time which elapses before foreign acids appear in the butter fat and before their disappearance after the cessation of dosing. The results obtained in the various experiments are shown graphically in Figs. 1 and 2.

(1) *Whole oil feeding* (Welcome 7, Fig. 1). Dosing was commenced at the rate of 2 ozs. twice daily, subsequently raised to 3 ozs. twice daily. The feeding of the cod-liver oil resulted in an extremely rapid rise in the absorption of the mixed butter acids. From an average value of 8·4 on the 3 days preceding dosing, $E/230$ for the mixed acids rose to 15·4 on the third day of dosing and to 35·2 on the seventh day of dosing. At this point a maximum value for the particular level of dosing appeared to have been reached. After 11 days on cod-liver oil, dosing was discontinued, with the result that the absorption of the acids fell almost as rapidly as it had risen. It may be noted, however, that the absorption did not quite return to the level observed before dosing began and also showed a number of minor fluctuations during the post-experimental period. Iodine value determinations on selected specimens of butter acids taken just before dosing and at the point when the absorption of the fatty acids had reached its maximum gave values of 37·6 and 50 respectively, the former value being about the usual level for winter acids.

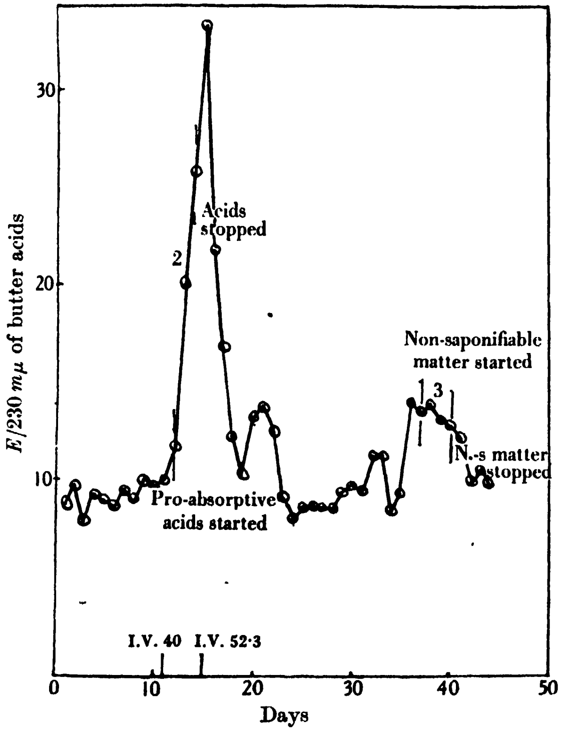

Fig. 2. Lottie 5. (2) Pro-absorptive acid feeding; (3) non-saponifiable matter feeding.

(2) "*Pro-absorptive*" *acid feeding* (Lottie 5, Fig. 2). The acids were prepared in bulk by a technique essentially similar to our usual routine method, the time taken over the saponification being kept short by ensuring that the alcoholic potash was at boiling-point before the addition of the cod-liver oil. Previous to dosing, $E/230$ for the mixed acids had shown a value not greater than 10 for 11

days. The cod-liver oil acids were given over a period of 3 days, in doses as follows:

p.m. 8. iii. 34	... ...	2 ozs.
a.m. 9. iii. 34	... ...	2 ozs.
p.m. 9. iii. 34	... ...	3 ozs.
a.m. 10. iii. 34	... ...	3 ozs.
p.m. 10. iii. 34	... ...	3 ozs.
a.m. 11. iii. 34	... ...	1·5 ozs.
	Total ...	14·5 ozs.

On the second day of dosing $E/230$ had risen to 20·0. The maximum value: 33·3, was reached one day after the cessation of dosing, after which the absorption fell rapidly. As in the previous experiments there were several minor fluctuations in the absorption during the post-experimental period. Iodine value before dosing 40, at point of maximum absorption 52·3.

(3) *Feeding of the non-saponifiable residue* (Lottie 5, Fig. 2). The crude unsaponifiable matter obtained by evaporation of the ether extracts of the soaps during the preparation of the acids was fed in doses calculated to correspond to the amounts of acids previously given. The period of dosing happened to correspond with a minor upward fluctuation in the "absorptive" power, probably related to previous acid feeding, otherwise the values obtained showed no divergence from the normal winter level.

(4) *Feeding experiments with "absorptive" acids* (Welcome 7, Fig. 1). The "pro-absorptive" acids ($E/230 = 8·6$), after preparation in the manner described above, were converted into the "absorptive" acids ($E/230 = 121$) by boiling for 37 hours with alcoholic potash. The doses given were the same as the doses of "pro-absorptive" acids described in (2), and 14·5 ozs. were fed over the period p.m. 23. iii. 34—a.m. 26. iii. 34.

The absorption of the butter acids rose rapidly, reaching a maximum value ($E/230 = 42·4$) 4 days after the commencement, and 2 days after the cessation, of dosing. The fall of the absorption from the maximum value was checked by a considerable upward fluctuation of several days' duration. Iodine values—before dosing 41·1, at point of maximum absorption 51·1.

The behaviour of the butter acids on treatment in vitro *by boiling with alcoholic potash.* In order to gain some insight into the proportion of "pro-absorptive" acids secreted in the butter fat, selected specimens from the group of butters described above were submitted to prolonged treatment with alcoholic potash, which was calculated to increase the absorption by significant amounts if "pro-absorptive" acids were present in quantity. The results obtained are given in Table IV. It will be seen that in no case was there more than a slight rise in the absorption as a result of this treatment. The behaviour of the acids was thus the same as that of the acids from normal winter or summer butter [Booth *et al.*, 1935].

Table IV.

	Butter fat acids		
Material given to cows	Time of treatment	$E/230$	I.V.
"Pro-absorptive" acids of cod-liver oil	2 mins.	33·3	52·3
	48 hrs.	26·5	46·5
Linseed oil	2 mins.	20	49·6
	48 hrs.	23·4	51
Rape oil	2 mins.	14·4	49·5
	48 hrs.	17·9	49·1

Differentiation between the "absorptive acids" and the "inhibitory substance" of the antimony trichloride test. Experiments, which need not be described in detail, have demonstrated that while the absorption of acids from butter fats of cows fed on linseed oil, cod-liver oil or sardine oil might be equal to or greatly exceed that of normal summer acids, the inhibitory power was invariably much lower.

DISCUSSION.

The conversion of "pro-absorptive" into "absorptive" acids. The above experiments demonstrate that certain fatty acids characterised by a slight absorption in the far ultra-violet ("pro-absorptive acids") can be converted *in vivo* and *in vitro* into acids having a much stronger absorption in the same region. The change can be brought about either by feeding the fatty acids (or the fat) to the cow or by treating the acids for long periods with caustic potash. For the present it must remain doubtful whether the changes occurring *in vivo* and *in vitro* are identical in all respects, but as a working hypothesis it will be assumed that the two processes are similar. Since the absorption of the butter acids from cows receiving ordinary diets or dosed with various oils shows no significant increase after prolonged treatment with potash, it seems probable that almost the whole of the ingested acids capable of transformation into the strongly absorbing substance either undergo the transformation or are not secreted into the milk.

Quantitative aspects of the transformation. The increase in the absorption of the fatty acids from the various oils brought about by treatment with caustic potash is self evident. On the other hand, the increased absorption of the fatty acids of the butter following the ingestion of the oils might seem to be explicable on other grounds than the transformation of "pro-absorptive" into "absorptive" acids. Two such alternative explanations may be advanced, but both can be excluded by examining the data quantitatively. In the first place it might be considered that the increased absorption was essentially due to the depression in the actual amount of butter fat secreted, on the assumption that the depression might take place at the expense of the non-absorbing constituents of the butter acids. Since however the depression seldom amounted to more than 30 %, while the absorption was sometimes increased 4–5 fold, it is obvious that this explanation would be inadequate. Secondly it might be argued that the increased absorption of the mixed butter acids over that of the ingested fat might be due not to an actual increase in the specific absorption of the ingested acids, but to the concentration in the milk fat of highly absorbing components present in low concentration in the ingested acids. This explanation also fails, however, since the increased absorption of the butter acids, calculated on weight basis, was always much greater than the absorption present in the ingested acids. To illustrate this point we may take the figures for Lottie 5, which was given a preparation of the acids of cod-liver oil in the "pro-absorptive" state. If we study the "absorption units" secreted in the butter acids during a 12-day period commencing with the 3 days during which the acids were administered, we may draw up the following balance sheet:

		$E/230 \times$ g. units
Absorption units secreted in butter acids. (Calculated by multiplying the daily butter acid yield in g. by the daily value for $E/230$.)		82,000
Allowance for basic absorption due to butter on winter scale (calculated by multiplying the total butter acid yield over the 12 days (5050 g.) by the average value for $E/230$ over 11 days preceding dosing (9·2))		46,000
Increase due to cod-liver oil acid feeding	=	36,000
Absorption present in cod-liver oil acids as ingested (411 g. having $E/230 = 8·6$)	=	3,500

The increase in absorption of the mixed acids of the butter fat was therefore about 10 times greater than the absorption of the ingested acids.

The nature of the change involved. At the present stage little can be said about the nature of the acids capable of showing increased absorption. Judging however from the limited experience of Dann and Moore [1933] it appears probable that the property is confined to acids having two or more unsaturated linkages. As to the cause of increased absorption, it appears probable that intramolecular changes take place. The possibility that oxidative changes might be involved appears to be ruled out by (1) iodine value determinations. In the *in vitro* experiments, the absorption rose to high values with little and in some cases no fall in the iodine value. This would indicate that only small amounts of oxidation products can have been formed. If these were responsible for the increased absorption, then the specific absorption must have been of an unusually high order. (2) Preliminary experiments have indicated that the "absorptive" acids are themselves unstable to oxidation and are therefore unlikely to be the end-products of an oxidative reaction. Thus when the mixed acids of cod-liver oil were first converted into the "absorptive" state by prolonged treatment with potash and then aerated for 24 hours at 95°, $E/230$ fell from 121 to 20·6. (3) Oxidative treatment of the "pro-absorptive" acids from cod-liver oil increases slightly the extinction coefficient at $230 m\mu$, but the characteristic band at that wave length is no longer evident, and the absorption is non-specific end absorption. The above evidence, of course, does not rule out the possibility of oxidative changes of the peroxide formation type, if those changes can be visualised as occurring without affecting the iodine-absorbing capacity of the unsaturated linkages.

The rapidity of transference of exogenous fats to the milk fat. As a point of secondary interest the above experiments provide a good illustration of the great rapidity with which foreign fats may be secreted in the milk fat, since when cod-liver oil, or preparations of its acid fraction, were administered, a distinct rise in the absorption of the butter acids could always be detected on the following day. The presence of the cod-liver oil acids in the butter fat could be readily observed by the formation of dark brown soaps on treatment of the fat with alcoholic potash. This is a property of the cod-liver oil acids, whereas the acids of butter fat form colourless soaps when obtained from cows subsisting under normal nutritional conditions.

SUMMARY.

1. In the mixed acids prepared from cod-liver oil, sardine oil, rape oil and linseed oil the absorption of radiation at wave length $230 m\mu$ is small. When the oils were fed to the cow the acids secreted in the butter fat possessed much greater absorption in this region.

2. A similar increase in absorption could be brought about by the prolonged boiling of the "pro-absorptive" acids with alcoholic potash.

3. The change from slightly absorbing ("pro-absorptive") acids to strongly absorbing acids is not an oxidative one; it is postulated that a molecular rearrangement occurs with production of an isomeric form.

Our thanks are due to Dr L. J. Harris and Prof. H. D. Kay for their valuable criticism.

APPENDIX.

Samples of two of the butters examined in the above experiments, and also two other samples not examined were sent to Prof. T. P. Hilditch who has kindly sent the following analyses: (Letter dated May 11th, 1934.)

Cow or cows ...	Gertie 3	Flora 16 Flora 20	Flora 16 Flora 20	Portia 3
Oil fed ...	None (control)	Rape oil	Linseed oil	Cod-liver oil
Milk collected from	p.m. 14. iii. 34	p.m. 13. iii. 34	p.m. 27. iii. 34	a.m. 17. iii. 34
to, inclusive	a.m. 16. iii. 34	a.m. 14. iii. 34	p.m. 28. iii. 34	p.m. 18. iii. 34
Reichert-Meissl value	33·6	28·5	28·9	16·0
Polenske value	3·3	1·8	2·1	1·4
Kirschner value	24·3	21·7	22·4	13·9
Saponification equivalent	239·2	251·2	249·3	264·2
Iodine value	34·5	44·5	46·0	51·7

Prof. Hilditch quoted these figures at the Meeting of the British Association at Aberdeen. He said, "These results indicate sufficiently clearly that intake of fatty oil by the cows may cause considerable modification of the milk fat; the influence of cod-liver oil on the character of the butter fat is especially marked in its diminution of the most volatile acids and the 50 % increase in mean unsaturation as compared with the control."

REFERENCES.

Booth, Kon, Dann and Moore (1935). *Biochem. J.* **29**, 133.
Dann and Moore (1933). *Biochem. J.* **27**, 1166.
Drummond, Channon, Coward, Golding, Mackintosh and Zilva (1924). *J. Agric. Sci.* **14**, 531.
Gillam, Heilbron, Hilditch and Morton (1931). *Biochem. J.* **25**, 30.

XIX. VITAMIN A AND CAROTENE.

XII. THE ELIMINATION OF VITAMIN A FROM THE LIVERS OF RATS PREVIOUSLY GIVEN MASSIVE DOSES OF VITAMIN A CONCENTRATE.

By ALAN WILLIAM DAVIES and THOMAS MOORE.

From the Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council.

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In a previous paper [Davies and Moore, 1934] we presented data showing that very high concentrations of vitamin A may be attained in the liver of the rat when large amounts of vitamin A concentrate are included in the diet. It was calculated that the amount of vitamin A stored might represent as much as 5 % of the dry weight of the liver, and be sufficient, if used economically, to satisfy the normal requirements of the animal for a theoretical period of about 200 years. The experiment to be described below was carried out to decide whether this theoretical economy would hold good in practice, or whether the reserve would be dissipated more rapidly. Under the particular experimental conditions adopted, the second alternative has been found to be correct.

EXPERIMENTAL.

32 female piebald rats were used. Until 10 months old they had been employed for breeding purposes, receiving a mixed dietary which included milk, liver (once weekly), green vegetables and carrots in sufficient amounts to permit the accumulation of a very high concentration of vitamin A in the liver even without further treatment. After retirement from breeding they were given the same mixed diet until 18 months old, when they were taken for use in the present experiment.

Preliminary "saturation" of the liver with vitamin A. With the exception of 3 animals, which were used in determining the level of the vitamin A reserve attained on the previous mixed diet, all the rats were now given a diet containing large amounts of vitamin A concentrate ("light white casein" 20 %, sugar 50 %, coconut oil containing vitamin A 25 %, salt mixture 5 %, dried yeast 10 %, wheat germ 5 %). The amount of vitamin A was adjusted so that 10 g. of diet contained 2 mg. of vitamin A concentrate (=6000 B.U.). Groups of three rats were killed at intervals, and the vitamin A reserves per g. of liver were determined by our usual method [Davies, 1933]. Although the feeding of this diet was continued for 12 weeks the liver reserves, as indicated by the mean values of the groups of three animals, rose only slightly (about 25 %) above the level attained on the mixed dietary. At this level a maximum value for these particular conditions of feeding appeared to have been reached.

The mean vitamin A reserves for each group of rats during this period are shown in the ascending part of the graph in Fig. 1. Although the values obtained in the individual rats of each group (see Table I) showed considerable variation from the mean value (usually about 33·3 %), the means for the groups gave a consistent series of values except for one group, which gave an anomalous mean indicated by point *A* in Fig. 1. One of the rats in this group gave an unexpectedly low result (1400 B.U. per g. as compared with the expected mean of 14,000 B.U. per g.).

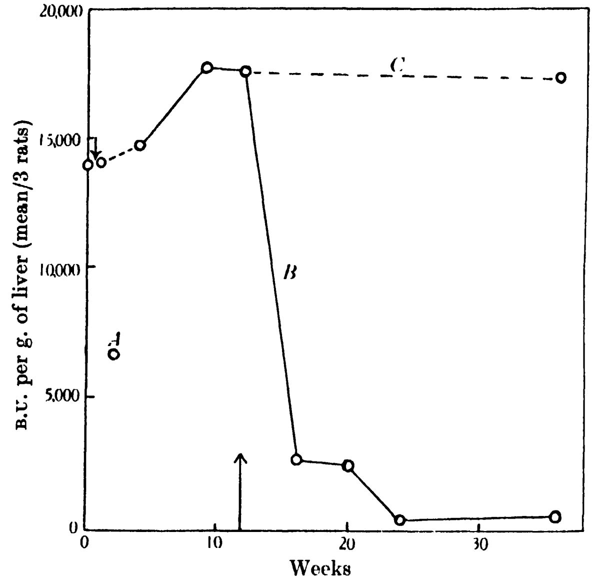

Fig. 1. *B*, Actual rate of decline. *C*, Calculated rate of decline of liver reserve assuming expenditure to be in accordance with physiological requirements. ↑ Rats given vitamin A-free diet. ↓ Rats given large amounts of vitamin A concentrate.

Table I.

Weeks from commencement of experiment	Weeks on vitamin A-free diet	Individual vitamin reserves. B.U. per g.			Mean vitamin A reserve B.U. per g.	Remarks
0	—	7,400	16,000	19,000	14,000	As taken from breeding stock
1	—	11,000	19,000	11,000	14,000	Vitamin A concentrate given
2	—	1,400	11,000	8,000	6,700	do.
4	—	1,100	16,000	17,500	15,000	do.
7	—	10,500	24,000	19,000	18,000	do.
12	—	14,000	20,000	19,000	18,000	do.
16	4	3,500	3,500	1,100	2,700	Vitamin A-free diet
20	8	2,500	2,500	2,400	2,500	do.
24	12	460	250	475	400	do.
36	24	600	500	470	500	do.

Dissipation of the vitamin A reserve on a diet deficient in vitamin A. The surviving rats were next given a diet having a composition similar to that recently proposed for use in vitamin A tests by Morgan[1] [1934] (extracted meat meal 10 %, coconut meal 30 %, ground rice 48 %, dried yeast 8 %, salt mixture 4 %, radiostol 1 drop per rat per week). This diet was found to be virtually free from vitamin A by independent growth tests with young rats.

[1] Our thanks are due to Lever Brothers, Ltd. for the gift of the special materials used in this diet.

Groups of three rats each were killed after they had received the vitamin A-free diet for periods of 4, 8, 12 and 24 weeks. The rapid fall in vitamin A reserves is shown in the descending part (*B*) of the graph in Fig. 1. After 4 weeks the mean liver reserve was found to have fallen to about $\frac{1}{6}$ of its previous value (2700 B.U. per g. as compared with 18,000 B.U. per g.), after 8 weeks the mean value (2500 B.U. per g.) was much the same as after 4 weeks, but after 12 weeks there was a further fall to 400 B.U. per g. The value obtained after 24 weeks (500 B.U. per g.), however, was virtually the same as that observed after 12 weeks, suggesting that a level of more stable storage had now been reached.

DISCUSSION.

The above experiment proves that superfluous reserves of vitamin A held in the liver may sometimes be eliminated at a much greater rate than that demanded to satisfy the known physiological requirements of the organism. In Fig. 1 a broken line (*C*) has been drawn to represent the rate of decrease in the mean vitamin A reserve per g. of liver which would have been expected if the vitamin had been used up at a rate consistent with physiological requirements, on the assumption that $0{\cdot}2\,\gamma$ (or about 1 B.U.) of pure vitamin A per g. of liver would be metabolised daily[1]. It will be seen that the rate of decrease of the mean reserve on this basis would be extremely slow, amounting to less than 1 % of the initial value during the 24 weeks under investigation. In contrast the mean reserve actually fell at the rate of about $100\,\gamma$ (=500 B.U.) of pure vitamin A per g. of liver per day during the first 4 weeks after the adoption of the vitamin A-free diet, while after 12 weeks about 98 % of the initial reserve had been eliminated. At this point there was a definite check in the rate of expenditure of the vitamin, since no further decrease could be detected after a further period of 12 weeks. It may be significant that the level of storage was now of the same order as the value of 250 B.U. per g. which we would consider a typical value for the healthy human being[2].

The above results stand in contrast with those obtained by Dann and Moore [1931], who used rats whose vitamin A reserves had been raised to high levels (up to 7000 B.U. per g.) as the result of their receiving red palm oil, a rich source of carotene. The vitamin A reserves of these rats after they had been restricted for 5–6 weeks to a diet deficient both in vitamin A and B were found to be as high (within the limits of experimental error) as those of similar rats which received the diet containing red palm oil up to the time of killing. Moore [1931] also failed to observe any spectacular reduction below the reputed initial values in the vitamin A reserves of two rats kept for periods of 10 and 68 days on a vitamin A-free diet, although after the longer period it is possible that a deterioration of up to 40 % may have occurred. From Fig. 1 the reduction during the same period in the present experiments must have amounted to 90 %.

Two explanations of the divergence between the present results and those obtained in previous work are possible. The more rapid decline of the vitamin A reserve in the present experiments may be due either to the use of a different form of vitamin A-free diet, or to the fact that the reserves were built up from alimentary supplies of preformed vitamin A instead of carotene which was used in the previous work. Both these possibilities are now under investigation, but in view of the time-consuming nature of the work we have thought it advisable to publish an account of our results at their present stage of development.

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[1] This estimate is more generous than that previously assumed [Moore, 1931].

[2] Data kindly made available by Dr A. Leese indicate that the vitamin A reserves per g. of liver in wild rats, as determined for specimens caught in the Edinburgh Zoological Gardens, are of about the same order as in the human being.

As a point of secondary interest arising in the course of the present investigation, we may draw attention to the extremely high level which the vitamin A reserve of the liver of the rat may reach in rats receiving an ordinary dietary containing carrots, green vegetables and moderate amounts of liver. According to our observations the concentration of vitamin A produced under such conditions is only slightly lower than that which may be obtained by the feeding of vitamin A concentrate.

SUMMARY.

Adult rats, already possessing high liver reserves of vitamin A, were given a diet containing massive doses of vitamin A concentrate. The liver reserves under these conditions reached levels having a mean value of about 18,000 B.U. per g., representing a supply sufficient to satisfy the theoretical requirement of the rat for about a century, if used up at a rate corresponding to the minimum physiological requirements.

When the rats were subsequently restricted to a diet free of vitamin A, however, a rapid elimination of vitamin A from the liver took place, the mean vitamin A reserve falling to 400 B.U. per g. after 12 weeks. At this level a condition of stable storage of the vitamin appeared to have been attained, since no further fall in the mean reserve could be detected after a further period of 12 weeks of restriction to the vitamin A-free diet.

These results stand in contrast with those obtained in previous experiments carried out in this laboratory, in which a similar rapid elimination of vitamin A from the liver was not observed. The possible causes of the difference are discussed.

Our thanks are due to Dr L. J. Harris for his valuable criticism.

REFERENCES.

Dann and Moore (1931). *Biochem. J.* **25**, 914.
Davies (1933). *Biochem. J.* **27**, 1770.
—— and Moore (1934). *Biochem. J.* **28**, 288.
Moore (1931). *Biochem. J.* **25**, 275.
Morgan (1934). *Biochem. J.* **28**, 1178.

XX. THE AVAILABLE CARBOHYDRATE OF FRUITS

DETERMINATION OF GLUCOSE, FRUCTOSE, SUCROSE AND STARCH

By ELSIE MAY WIDDOWSON and
ROBERT ALEXANDER McCANCE.

From the Biochemical Department, King's College Hospital, London.

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It is now generally recognised that determinations of available carbohydrate in fruits and vegetables are of much more value for dietetic purposes than those of total carbohydrate, whether the latter have been determined by acid hydrolysis or calculated by difference.

Figures for the available carbohydrate of the fruits and vegetables commonly eaten in this country were given by McCance and Lawrence [1929]. An acid hydrolysis was used, followed by a determination of "total" reducing power. Realising, however, that this reduction was partly due to the products from easily hydrolysable pentosans and other non-fermentable sugars, McCance and Lawrence determined these constituents separately and deducted their reducing value from that of the total sugar. The remainder they considered to be available carbohydrate. Their figures in some instances are too low because the strong acid employed and the prolonged time of boiling led to a destruction of a considerable proportion of the fructose. They mentioned that something of this sort was taking place, but considered that for practical purposes the error could be neglected.

A study of the available carbohydrates of fresh fruits has now been made by the most reliable methods which can be obtained. Sucrose, fructose, glucose and starch have each been determined separately. Since these are generally considered to be the only fruit carbohydrates which are absorbed as sugars from the gut, their sum constitutes the available carbohydrate of the fruit. Small amounts of maltose may be present in starchy fruits such as the banana. If this is the case, the error so introduced must be insignificant, since determinations of glucose and fructose by two independent methods have been found to agree. From the nature of the methods employed this would not have been the case if appreciable amounts of maltose had been present (*vide infra*).

Fresh, raw fruits only have been used, and all determinations have been carried out on the edible portion.

METHODS.

Preparation of the fruit. Almost all the fruit has been obtained from the Amalgamated Fruiterers and the Army and Navy Stores. Each variety of English and foreign fruit came from at least four, and usually from six, different sources and was known therefore to have been grown under various soil and climatic conditions. Thus a representative sample of the fruit was obtained. The analyses were all carried out during the 1934 season.

The method used for the preparation and extraction of the fruit was similar to that described by Archbold [1932] for apples. About 3 lbs. of small fruit and 6–8 lbs. of larger varieties were prepared as for eating, and the proportion of waste was determined. The edible portion was then cut up finely and well mixed. Water was determined on duplicate samples of 50 g. by drying to constant weight at 50°.

Duplicate portions of 100 g. were extracted with about 200 ml. of cold 95 % alcohol overnight. The tissue was next extracted with hot 80 % alcohol in a Soxhlet apparatus for about 16 hours, and the alcohol from the united extracts was evaporated off under reduced pressure at a temperature always below 30°. The residue was made up to 200 ml. in a graduated flask (Solution A). Free acid was determined on 20 ml. of this extract by titration with $N/10$ NaOH. Phenolphthalein was used as indicator.

Determination of reducing sugars. 50–100 ml. of Solution A were measured into a graduated 500 ml. flask, diluted with water, almost neutralised with $N/10$ NaOH and cleared with basic lead acetate and saturated sodium phosphate solutions [Archbold and Widdowson, 1931]. The solution was made up to volume and filtered. Reducing sugars were determined in this cleared filtrate (Solution B) by Lane and Eynon's [1923] copper titration method, in which methylene blue is used as an internal indicator. Dilution of Solution B was sometimes necessary before the estimations could be carried out.

Determination of sucrose. Total sugar was estimated in Solution B after inversion with 10 % citric acid for 10 mins. at 100°. The difference between the percentage of total sugar and of reducing sugar, calculated from Lane and Eynon's invert sugar table, gave the percentage of sucrose in the fruit. Preliminary tests on various fruits indicated that the values for sucrose obtained in this way were the same as those obtained after inversion with invertase (see Table I).

Table I. *Sucrose content of fruits after inversion by citric acid and by invertase.*

Results expressed as percentage in fresh material.

Fruit	Sucrose by citric acid	Sucrose by invertase
Gooseberry	0·23	0·22
Banana	4·2	4·3
Apple	2·6	2·7
Lemon	0·34	0·28
Strawberry	1·32	1·32
Tomato	0·0	0·0

Determination of the fructose/glucose ratio. Before carrying out the iodimetric procedure for the determination of the fructose/glucose ratio, it was necessary to decolorise solution B. 100 ml. were boiled with 0·5 g. B.D.H., decolorising charcoal for 1 min. and filtered. This process was repeated until the liquid was colourless. The charcoal was thoroughly washed with boiling water and the filtrate and washings made up to 200 ml. (Solution C). It has previously been shown that this procedure removes only negligible amounts of sugars from solution [Widdowson, 1931]. The iodine value of solution C was determined exactly as described by Archbold and Widdowson [1931]. The fructose/glucose ratio was calculated by solving the simultaneous equations obtained from the iodimetric determination and from the estimation of the reducing power of solution B by Lane and Eynon's copper method.

Fructose has also been determined directly in Solution B in a number of different fruits by the method described by Oppel [1930]. This is a colorimetric method based upon the colour developed when fructose is heated with diphenylamine and strong hydrochloric acid. Since the acid hydrolyses any sucrose present, the value obtained for fructose is the sum of the free fructose and the fructose derived from the sucrose. A standard solution of sucrose was used for the comparisons. Table II shows the amounts of fructose (including free fructose and sucrose-fructose) in a variety of fruits as estimated by the two independent methods.

Table II. *Total fructose in fresh fruits estimated by Fehling's solution and iodine and by diphenylamine.*

Results expressed as percentage of fructose in fresh fruit.

Fruit	Fehling's solution and iodimetric method	Diphenylamine method
Pear	7·9	7·7
Blackberry	3·6	3·6
Grapefruit	2·3	2·6
Pineapple	4·9	5·0
Orange	4·0	4·1
Grape	8·0	7·9
Cranberry	0·8	1·1
Date	29·9	29·4
Banana	6·9	7·0

It will be observed that in most cases agreement is very close. In the case of cranberries the slightly higher results given by the diphenylamine method can be explained by the fact that in this fruit there is a large excess of glucose with which the reagent reacts to a small extent.

Determination of starch. The fresh fruit was in every case tested with iodine in order to detect the presence of starch. Of all the fruits examined, starch was found to be present only in the banana, apple, pear and tomato. The residue remaining after alcoholic extraction of these fruits was dried at 100° overnight and weighed.

The dried residue from the banana was ground up in a mortar and sifted. Duplicate samples of 0·5 g. were weighed out and soaked in 10 ml. water for several hours. It was impossible to grind the residues from the apple, pear and tomato owing to their tough and leathery nature. These were therefore weighed out without grinding and soaked in water as just described. The wet material in each case was ground up in a mortar to a fine pulp and transferred quantitatively to a small flask with a further 40 ml. of water, which was then boiled.

Takadiastase was used to hydrolyse the starch, and the reducing power was determined on the filtrate after clearing with basic lead acetate by Hanes's [1929] modification of the Hagedorn and Jensen ferricyanide technique. Full details have been described by Widdowson [1932].

RESULTS.

The results of the analyses are shown in Table III.

Values are given for the percentages of glucose, fructose, sucrose and starch in the edible portion of the fruits, and the total available carbohydrate is calculated as the sum of these 4 individual carbohydrates. The water content of the fruits is also shown and their titratable acidity. For each fruit a factor is also given. By

Table III. *Available carbohydrate content of fruits.*

Fruit	Percentage in edible portion						Titratable acidity ml. $N/10$ NaOH ≡ 100 g. fruit	Conversion factor	Nature of edible (analysed) portion and waste
	Glucose	Fructose	Sucrose	Starch	Total available carbohydrate	Water			
Apples, Empire eating	2·20	7·25	2·70	0·02	12·17	84·1	67	0·75	Flesh analysed. Skin and "core" discarded
Apples, English eating	1·72	6·08	3·62	0·30	11·72	84·5	82	0·79	Flesh analysed. Skin and "core" discarded
Apples, English cooking	1·82	5·01	2·40	0·35	9·58	85·6	189	0·81	Flesh analysed. Skin and "core" discarded
Apricots	1·93	0·37	4·35	0	6·65	86·6	230	0·92	Flesh and skin analysed. Stones discarded
Bananas	5·82	3·78	6·58	3·02	19·20	70·7	68	0·57	Flesh analysed. Skin discarded as waste
Blackberries, mixed hedge and cultivated	3·24	2·88	0·24	0	6·36	82·0	139	1·0	Whole fruit analysed
Cherries, eating	4·70	7·24	0	0	11·94	81·5	113	0·87	Flesh and skin analysed. Stalks and stones discarded
Cherries, cooking	5·50	6·11	0	0	11·61	79·8	145	0·84	Flesh and skin analysed. Stalks and stones discarded
Cranberries	2·66	0·74	0·14	0	3·54	87·0	389	1·0	Whole fruit analysed
Currants, black	2·35	3·67	0·62	0	6·64	77·4	443	0·98	Flesh, skin and pips analysed. Stalks discarded
Currants, red	2·28	1·93	0·15	0	4·36	82·8	350	0·97	Flesh, skin and pips analysed. Stalks discarded
Currants, white	3·03	2·56	0	0	5·59	83·3	223	0·96	Flesh, skin and pips analysed. Stalks discarded
Damsons	5·22	3·42	0·96	0	9·60	77·5	307	0·90	Flesh and skin analysed. Stones and stalks discarded
Dates	32·00	23·70	8·20	0	63·90	14·6	65	0·86	Flesh and skin analysed. Stones discarded
Figs, green	5·54	4·00	0	0	9·54	84·6	50	0·98	Flesh, skin and pips analysed. Stalks discarded
Gooseberries, green	1·47	1·70	0·25	0	3·42	89·9	237	0·99	Flesh, skin and pips analysed. "Tops and tails" discarded
Gooseberries, ripe	4·40	4·10	0·71	0	9·21	83·71	245	0·99	Flesh, skin and pips analysed. "Tops and tails" discarded
Grapes, black	8·20	7·28	0	0	15·48	80·7	96	0·81	Flesh analysed. Skin, pips and stalks discarded
Grapes, white	8·12	8·01	0	0	16·13	79·3	58	0·95	Flesh and skin analysed. Stalks and pips discarded
Grapefruit	1·95	1·24	2·14	0	5·33	90·7	203	0·48	Flesh only analysed. Skin, pith and pips discarded
Greengages	5·00	2·60	4·16	0	11·76	78·2	219	0·95	Flesh and skin analysed. Stones and stalks discarded
Lemons, whole	1·40	1·35	0·41	0	3·16	85·2	213	1·0	Whole fruit analysed including skin
Lemon juice	0·52	0·92	0·18	0	1·62	91·3	947	0·36	Juice only analysed
Loganberries	1·94	1·26	0·23	0	3·43	85·0	332	1·0	Whole fruit analysed
Melons, Cantaloupe	1·16	0·83	3·26	0	5·25	93·6	17	0·59	Flesh analysed. Skin and pips discarded
Melons, yellow	2·09	1·52	1·43	0	5·04	94·2	19	0·59	Flesh analysed. Skin and pips discarded
Mulberries	4·44	3·64	0	0	8·08	85·0	247	1·0	Whole fruit analysed
Oranges	2·51	1·81	4·19	0	8·51	86·1	102	0·75	Flesh only analysed. Outer skin and pips discarded
Orange juice	2·36	2·38	4·70	0	9·44	87·7	134	0·46	Juice only analysed
Peaches	1·47	0·93	6·66	0	9·06	86·7	114	0·87	Flesh and skin analysed. Stones discarded
Pears, Empire eating	3·47	6·28	1·04	0	10·79	83·0	25	0·69	Flesh only analysed. Skin and "core" discarded
Pears, English eating	2·44	7·00	0·98	0	10·42	83·4	44	0·75	Flesh only analysed. Skin and "core" discarded
Pears, English cooking	2·18	6·00	1·12	0·04	9·34	83·0	57	0·77	Flesh only analysed. Skin and "core" discarded
Pineapple, fresh	2·32	1·42	7·89	0	11·63	84·3	169	0·54	Flesh analysed. Skin and "core" discarded
Plums, dessert	4·00	1·34	4·26	0	9·60	84·1	252	0·95	Flesh and skin analysed. Stones and stalks discarded
Plums, cooking	3·50	1·27	1·45	0	6·22	85·1	356	0·91	Flesh and skin analysed. Stones and stalks discarded
Pomegranate juice	5·46	6·14	0	0	11·60	85·4	146	0·56	Juice only analysed
Raspberries	2·26	2·39	0·96	0	5·61	83·2	275	1·0	Whole fruit analysed
Strawberries	2·59	2·32	1·30	0	6·21	88·9	125	0·97	Flesh and pips analysed. Stalks discarded
Tomatoes	1·63	1·17	0	0·02	2·82	93·4	73	0·99	Flesh, skin and pips analysed. Stalks discarded

multiplying the percentages in the edible portion by the factor, the quantities which would be obtained from the food as purchased can be derived. The final column shows the parts of the fruits which were included in the edible portion.

DISCUSSION.

It will be noticed that only a very small proportion of the ripe fruits contains starch, and even in these cases the amount present is negligible. Bananas are exceptional. 16 % of the total available carbohydrate was present as starch in the samples examined, *i.e.* 3 % of starch in the fresh fruit. It is a well-known fact that the starch/sugar ratio in the banana depends upon the degree of ripeness of the fruit, and quite different results would no doubt have been obtained had unripe or over-ripe fruits been analysed.

Most of the fruits examined contain approximately equal amounts of free fructose and glucose, but apples and pears have much more fructose than glucose, and plums, damsons, apricots and similar fruits and also cranberries contain more free glucose than fructose. Grapes contain glucose and fructose in nearly equal amounts.

A few fruits, notably cherries, grapes, figs, tomatoes, pomegranates and mulberries, contain no sucrose at all. In one or two cases, *e.g.* apricots and peaches, sucrose constitutes the greater part of the total sugar present.

Comparison of the present results with those obtained after acid hydrolysis.

It has been stated earlier in the paper that McCance and Lawrence's [1929] figures for the carbohydrate contents of fruits were too low, owing to a destruction of part of the fructose during the acid hydrolysis. This is shown to be the case by the fact that on boiling a 2 % solution of fructose with hydrochloric acid (5 % by volume), 28 % of the total fructose present was destroyed during the first hour and 48 % after 2 hours. Glucose was almost unchanged by this treatment.

Samples of seven of the fruits used in the present investigation were boiled with 5 % acid for 2 hours and estimated as described by McCance and Lawrence. The results of these determinations are shown in Table IV, Column 1. Column 2 shows the pentose content of fruits and Column 3 the available carbohydrate after deducting the pentose sugars [McCance and Lawrence, 1929]. Taking the

Table IV. *Comparison of available carbohydrate in fruits as determined in the present investigation and after acid hydrolysis.*

Results expressed as percentage of edible portion of fresh fruit.

Fruit	Direct determination of carbohydrate after acid hydrolysis	Pentose	Carbohydrate corrected for pentose	Carbohydrate corrected for pentose and fructose	Sum of glucose, fructose, sucrose and starch
Blackberries*	8·4	1·8	6·6	8·4	7·6
Cranberries	4·0	0·9	3·1	3·5	3·5
Damsons	9·5	2·2	7·3	9·3	9·6
Grapes, black	12·5	0·6	11·9	15·5	15·5
Grapefruit	5·3	1·1	4·2	5·3	5·3
Orange juice	6·9	0	6·9	9·2	9·4
Pears, cooking	7·9	1·7	6·2	9·5	9·3

* Blackberries have been analysed on 3 occasions. The figure in Table III is the mean of all three analyses, while the figure in this table is the result of one of them.

figures for fructose found in the present investigation (free fructose + sucrose-fructose), and assuming that 50 % of this had been destroyed during the 2 hours' hydrolysis, the figures in Column 3 have been corrected, and the results are shown in Column 4. It will be seen that in almost every case they agree very closely with the present results for total available carbohydrate calculated as the sum of the glucose, fructose, sucrose and starch (Column 5). It is evident therefore, that McCance and Lawrence were justified in deducting the values for pentose sugars, and that their figures for vegetables which contain no fructose were probably correct. Further, McCance and Lawrence's published results were not so much too low as the differences between Columns 2 and 4 suggest, since they took the mean of a 1- and 2-hour hydrolysis, and the present figures were obtained after treating for a full 2 hours with the acid.

SUMMARY.

The glucose, fructose, sucrose and starch in 41 varieties of fruit have been determined.

Sucrose was estimated by the increase in reducing power after inversion.

The glucose/fructose ratio was calculated by solving the simultaneous equations obtained from an iodimetric determination and from the estimation of reducing power by Fehling's solution. Fructose was also determined by diphenylamine with concordant results.

Starch was estimated as glucose and maltose after digestion with takadiastase.

The sum of these four individual carbohydrates was taken to represent the available carbohydrate of the fruit.

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REFERENCES.

Archbold (1932). *Ann. Bot.* **46**, 407.
—— and Widdowson (1931). *Biochem. J.* **25**, 101.
Hanes (1929). *Biochem. J.* **23**, 99.
Lane and Eynon (1923). *J. Soc. Chem. Ind.* **42**, 132 T.
McCance and Lawrence (1929). *Med. Res. Coun. Special Report, Series.* No. 135.
Oppel (1930). *Biochem. Z.* **229**, 85.
Widdowson (1931). *Biochem. J.* **25**, 863.
—— (1932). *Ann. Bot.* **46**, 597.

XXI. THE EFFECT OF POTASSIUM ON THE GLUCOLYSIS OF BRAIN TISSUE WITH REFERENCE TO THE PASTEUR EFFECT.

By CHARLES AMOS ASHFORD[1] and
KENDAL CARTWRIGHT DIXON.

From the Biochemical Laboratory, Cambridge.

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Various explanations have hitherto been put forward as to the mechanism of the Pasteur effect. Meyerhof *et al.* [1925] and others have suggested that the lactic acid formed in anaerobiosis is also formed under aerobic conditions, but here removed by oxidation and perhaps resynthesis. Another explanation has been given by Lipmann [1933], who considers that oxygen interferes with the activity of the fermentative or glucolytic enzyme and thus spares glucolysis. It seemed also possible that one of the reactants in the lactic acid-forming reaction (as demonstrated by the work of Embden *et al.* [1933] and Meyerhof and Kieseling [1933] in the case of muscle) might be removed by oxygen. Thus, assuming glucose to split into two molecules a and b which react together to give lactic acid, we have glucose $= a + b = 2$ lactic acid. If either a or b is removed by oxygen, lactic acid formation is lessened, and furthermore one atom of oxygen could prevent the formation of 2 molecules of lactic acid. That is, one molecule of oxygen absorbed prevents 4 molecules of lactic acid from accumulating, and in this way a maximum Meyerhof quotient of 4 could be explained.

In the original project, it was decided in the first place to investigate the effect of adding α-glycerophosphate and pyruvate (which was already known [Bumm *et al.*, 1933; Mendel *et al.*, 1931] to increase anaerobic glucolysis) to tissues in which oxygen was exercising its sparing action on glucolysis. If either of these substances occurred in the normal glucolysis and was removed by oxygen, addition of this substance during aerobiosis might restore and increase the aerobic glucolytic rate.

It was necessary to use for this purpose a tissue with low aerobic glucolysis, and for this purpose brain slices were selected for a start, although it is not believed [Ashford and Holmes, 1929; Ashford, 1933; 1934] that the Embden-Meyerhof scheme for glucolysis obtains in brain tissue. The work reported here is the outcome of these experiments.

Methods.

The tissue employed was the cortex of rabbit's brain. Slices of this were cut immediately after the rabbit was killed. These slices were immersed in 10 ml. of liquid which contained Ringer in normal concentration and 0·025M sodium bicarbonate. In this solution glucose was present in a concentration of 0·2 %, also any substance whose effect on glucolysis was to be determined. This volume of fluid containing the slices was contained in flasks provided with glass stoppers (as described in a previous paper [Ashford, 1934]). Oxygen or nitrogen containing 5 % CO_2 was bubbled through the liquid in which the slices were immersed for about 20 mins. Then the taps were closed and the flasks with their contained slices under physiological conditions were shaken in a bath at 37° for periods of 1, 2 or 3 hours. At the end of the period of shaking the flasks were removed from the bath, and 3 ml. of 20 % trichloroacetic acid were added to the contents of each which were quantitatively filtered and washed into a 50 ml. volumetric flask after standing about 10 mins. The slices contained in each shaking flask were transferred from the filter-paper into weighing bottles and their dry weight determined.

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[1] Beit Memorial Research Fellow.

The filtrates were made up to 50 ml. with water, and their lactic acid contents were determined, after treatment with copper lime, by Friedemann, Cotonio and Shaffer's [1927] method.

EXPERIMENTAL.

The effect of potassium α-glycerophosphate on the aerobic and anaerobic glucolysis was first studied. This salt was prepared in approximately 4 % solution by treatment of Merck's "Calcium glycerophosphoricum neutrale" with potassium oxalate at 0°. The calcium oxalate was centrifuged off, and the resulting filtrate was tested for freedom from both calcium and oxalate ions by the addition of small quantities of potassium oxalate or calcium chloride. The results of a number of experiments are shown in Table I. It will be seen that the addition of potassium α-glycerophosphate has markedly increased the aerobic glucolysis while at the same time the anaerobic glucolysis is so depressed as to be below the level of the aerobic in the presence of the potassium α-glycerophosphate.

Table I. *Effect of potassium glycerophosphates on anaerobic and aerobic glucolysis.*

All values in mg. lactic acid per g. dry tissue per hour.
The salts used had a final concentration of about 0·7 %.

		Nitrogen		Oxygen		
Exp. No.	Time	Glucose alone	Glucose + glycerophosphate	Glucose alone	Glucose + glycerophosphate	Remarks
1	3 hours	41·0	17·0	15·0	57·0	α-salt
2	3 hours	57·0	25·0	15·5	45·0	α-salt
3	2 hours	66·5	26·0	—	—	α-salt
4	2 hours	59·5	22·0	—	—	β-salt
5	2 hours 10 mins.	62·0	25·6	23·1	57·6	α-salt

The next step was to try the effect of potassium β-glycerophosphate and also the effects of the sodium salts (Tables I and II). In Table I it is shown that

Table II. *Effect of sodium glycerophosphates on anaerobic and aerobic glucolysis.*

All values in mg. lactic acid per g. dry tissue per hour.

Exp. No.	Time hours	Gas	Glucose alone	Glucose + α-glycero-phosphate	Glucose + β-glycero-phosphate	Remarks
1	3	N_2	58·0	85·0	81·0	0·4 % salts
		O_2	26·0	27·0	21·0	,, ,,
2	3	N_2	65·0	61·0	54(?)	0·6 % salts
		O_2	17·0	27·0	24·0	,, ,,
3	3	O_2	12(?)	27·7	21·7	0·9 % salts
		O_2	15·4	16·5	—	0·3 % ,,
4	2	N_2	53·5	—	61·0	0·3 % salts

potassium β-glycerophosphate has the same inhibitory effect on the anaerobic glucolysis as the α-salt. The sodium salts, however, have no effect, except in one case perhaps causing a slight rise in aerobic glucolysis, but not by any means of the same order as that caused by the potassium salt (Table II).

A further step was to see how much of this effect was due to the potassium ion or whether the glycerophosphate was the determining factor. To test this parallel experiments were carried out using potassium α-glycerophosphate and a solution of potassium choride against one another. Table III shows the result of the experiment and makes it clear that this ability to stimulate aerobic glucolysis and to inhibit anaerobic glucolysis in brain is a property of the potassium ion. It has already been noted [Ashford, 1934] that the presence of a potassium phosphate buffer reduces the anaerobic glucolytic rate in brain tissue.

Table III. *Comparison of effects of potassium α-glycerophosphate and potassium chloride on aerobic and anaerobic glucolysis.*

All values in mg. lactic acid per g. dry tissue per hour.
Duration of experiment—2 hours.

Gas	Glucose alone	Glucose + 1·4 % K α-glycero-phosphate	Glucose + 0·9 % KCl
N_2	50	16	9
O_2	13	75	51

Experiments were next performed to determine whether sodium chloride had any similar effect to potassium chloride. It is shown however in Table IV that sodium chloride has no significant effect on glucolysis, while experiments with the same concentration of potassium chloride show the effect in a marked manner.

Table IV. *Comparison of the effects of potassium and sodium chlorides on aerobic and anaerobic glucolysis.*

All values in mg. lactic acid per g. dry weight per hour.
Duration of experiment – 2 hours.

Gas	Glucose alone	Glucose + 0·1*M* NaCl	Glucose + 0·1*M* KCl
N_2	75	66	23
O_2	18	22	61

The effect of variation in concentration of potassium chloride was next studied. In Table V, the results of four experiments with 5 different concentrations of potassium chloride are shown. (It was unfortunately not possible to perform aerobic and anaerobic experiments on the same brain owing to the im-

Table V. *Effects of different concentrations of potassium chloride on aerobic and anaerobic glucolysis.*

All values in mg. lactic acid per g. dry tissue per hour.
Duration of experiments—2 hours.

Gas	Glucose + no KCl	Glucose + 0·005*M*	Glucose + 0·02*M*	Glucose + 0·1*M*	Glucose + 0·3*M*	Glucose + 0·5*M*	Date
N_2	54	67	41	14	13	8	31. vii. 34
N_2	53	55	30	10	9	14	8. viii. 34
O_2	17	17	30	49	35	19	30. vii. 34
O_2	19	16	25	58	32	24	3. viii. 34

possibility of obtaining sufficient tissue.) The results are shown in Fig. 1, where the glucolysis is plotted against the logarithm of the potassium chloride concentration, each experiment being plotted as a separate curve. It brings out clearly that the effect of potassium is greatest at a concentration of about $0{\cdot}1M$. The aerobic glucolysis has been increased 200 % while the anaerobic has been reduced 75 %, and it is worthy of note that at this concentration the aerobic glucolysis is 4 or 5 times as large as the anaerobic.

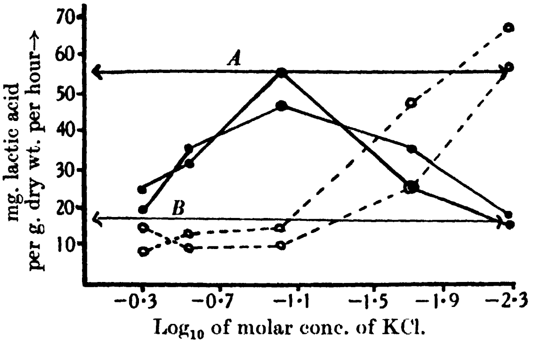

Fig. 1. Relation between anaerobic and aerobic glucolysis and potassium chloride. *A*, average level anaerobic, no KCl. *B*, average level aerobic, no KCl.

●—●—● Aerobic glucolysis. ○– –○– –○ Anaerobic glucolysis.

It would appear probable that at the concentration of normal Ringer, potassium does not exert this effect. Lasnitzki [1933] has shown that the absence of potassium and to some extent of calcium from the incubation fluid markedly reduces anaerobic glycolysis in tumour, but he did not study the aerobic effect. Beebe [1904] and Clowes and Frisbie [1905] have reported high values for potassium content of malignant tumours and these were often associated with low calcium content. Since high aerobic glucolysis is shown by tumours it was decided to ascertain whether glucolysis in brain was affected by the absence of calcium from the Ringer (Table VI). It will be seen that the marked effect of high potassium concentration is not shown by lack of calcium.

Table VI. *Effect of calcium-free Ringer on glucolysis.*

Gas	Medium (containing 0·2 % glucose)	mg. lactic acid per g. dry wt. per hour
N_2	Ringer	65
N_2	Ca-free Ringer	53
O_2	Ringer	13
O_2	Ca-free Ringer	20

It seemed of importance to ascertain whether the addition of pyruvate to brain slices under anaerobic or aerobic conditions would increase the glucolysis and also if the addition of co-ferment T would in any way modify the inhibitory effect of potassium salts on the anaerobic glucolysis, since it has been shown by the work of Mendel *et al.* [1931] and by that of Bumm *et al.* [1933] that in the phenomenon of inhibition by glyceraldehyde, in the case of tumour slices, this inhibition is removed by pyruvate and by co-ferment T. It will be seen from Table VII that in these experiments pyruvate does not increase the aerobic or anaerobic glucolysis. The absence of effect on the latter process is readily ex-

Table VII. *Effects of pyruvate and of pyruvate in the presence of KCl on glucolysis.*

Exp.	Medium	mg. lactic acid per g. dry tissue per hour Anaerobic	 Aerobic
1	Normal Ringer + glucose	83	22
	„ „ + 0·01M pyruvate	81	24
	„ „ + 0·001M „	—	26
2	Normal Ringer + glucose	70	—
	„ „ + 0·1M KCl	18	—
	„ „ + 0·002M pyruvate	69	—
	„ „ + „ „ + 0·1M KCl	20	—

plained by the fact that the glucolytic rate is already very high and is not further increased by addition of co-ferment T. The result is not therefore out of harmony with the work of the authors just cited, since in their experiments with brain tissue the anaerobic rates without pyruvate were usually of a somewhat low order. The absence of effect on the aerobic process is in agreement with Bumm *et al.* [1933]. Evidently oxidative removal of pyruvate does not influence lactic acid formation, since the addition of pyruvate has not increased the aerobic glucolysis. Table VII also shows that pyruvate has no significant effect on the inhibition of anaerobic glucolysis by potassium.

It has previously been shown that *dl*-glyceraldehyde markedly inhibits the anaerobic glucolysis of tumour [Mendel, 1929]. It was decided to see if the large aerobic glucolysis of brain slices in the presence of added potassium chloride was affected by glyceraldehyde. It was considered that if another mechanism of lactic acid formation in aerobiosis were operative here, this mechanism might not be inhibited by glyceraldehyde, as is the normal anaerobic mechanism. The results of experiments to test this point are shown in Table VIII together with the results of an experiment on the effect of glyceraldehyde on both normal anaerobic and aerobic glucolysis. It is apparent that the high aerobic glucolysis in the presence of potassium salts is inhibited by glyceraldehyde as is the normal anaerobic glucolysis. Further it appears that the aerobic glucolysis in the absence of potassium is also inhibited, though, owing to the small absolute value of the aerobic glucolysis, this can hardly be said to be proved.

Table VIII. *Effects of* dl-*glyceraldehyde and of glyceraldehyde with KCl on glucolysis.*

Exp.	Medium	mg. lactic acid per g. dry tissue per hour Anaerobic	 Aerobic
1	Normal Ringer + glucose	55	11
	„ „ + 0·03 % glyceraldehyde	4	6
2	Normal Ringer + glucose	—	15
	„ „ + 0·03 % glyceraldehyde	—	6
	„ „ + 0·1M KCl	—	40
	„ „ + „ „ + 0·03 % glyceraldehyde	—	6

Reversibility of the potassium effect.

The question as to whether these effects of addition of potassium are reversible has been investigated. The experiments are recorded in tabular form in

Table IX. *Experiment to test the reversibility of the potassium effect.*

All fluids contained 0·2 % glucose.

No. of flask	Gas	Media and procedure	mg. lactic acid per g. dry tissue per hour
1	N_2	Ringer + 0·1*M* KCl, stopped after one hour	19
2	O_2	As flask 1	60
3	N_2 then O_2	Ringer + 0·1*M* KCl, for one hour in N_2, then O_2 bubbled through and incubated for one hour further	17 (in 2nd hour)
4	N_2	Ringer + 0·1*M* KCl for one hour, then slices to flask 5	19
5	N_2	Ringer (no KCl) + slices from 4, for one more hour	4
6	O_2	Ringer + 0·1*M* KCl, for one hour, then slices transferred to flask 7	44
7	O_2	Ringer (no KCl) + slices from 6 for one more hour	20

Table IX, the numbers of the glass flasks in which the slices were incubated and their contents, gaseous and fluid are given, and the glucolysis is also recorded. From the value of the glucolysis in flask No. 3, we see that admitting O_2 to slices which have been in N_2 and KCl for 1 hour fails now to establish a high glucolytic rate, since only 36 mg. of lactic acid per g. dry weight are formed in No. 3, and 19 mg. of this have been formed in the first hour as in No. 1, 17 mg. having been formed in the second hour in O_2 and KCl. Also the high normal anaerobic glucolysis is not re-established in normal Ringer after the tissues have been incubated anaerobically for 1 hour with added KCl; this is seen from Nos. 4 and 5. On transferring tissues from Ringer with KCl, in which they have been for 1 hour, to normal Ringer, all under aerobic conditions, we see that the aerobic glucolysis now falls approximately to the normal level; this is shown by the figures for Nos. 6 and 7.

It appeared necessary to ascertain if the glucolysis in the second hour when tissues are transferred to another medium, is as high as in the first hour in the original medium, accordingly the experiment recorded in Table X was performed.

Table X. *Effect on glucolytic rates of transferring tissues from one medium to another.*

All fluids contained 0·2 % glucose.

No. of flask	Gas	Media and procedure	mg. lactic acid per g. dry tissue per hour
1	N_2	Ringer, then tissues to flask 2 after one hour	73
2	N_2	Ringer, slices from 1 for another hour	56
3	N_2	Ringer + 0·1*M* KCl for one hour	31
4	N_2	Ringer + 0·1*M* KCl + slices from flask 3 for a further hour	13
5	N_2	Ringer + 0·1*M* KCl for one hour	28
6	O_2	Ringer + 0·1*M* KCl + slices from flask 5 for a further hour	10

Nos. 1 and 2 show that lactic acid formation in normal Ringer falls off to some extent on transferring the tissues from their original fluid. Nos. 3 and 4 demonstrate a falling off in KCl under anaerobic conditions, while Nos. 5 and 6 show again that admission of O_2 to slices which have been an hour in KCl and N_2 cannot restore the high glucolytic rate seen in KCl and O_2.

From these experiments it is seen that the inhibition caused by KCl and N_2 is associated, at any rate after exposure for 1 hour to 0·1*M* KCl, with an irreversible change in the cells, so that they cannot regain a high glucolysis. Also it appears that tissues placed in normal Ringer after having been incubated in O_2 and KCl can now only show their normal low aerobic glucolysis (Nos. 5 and 6). It has been shown that the glucolysis in the second hour in O_2 and KCl is of the same order as in the first, this is seen from figures showing the glucolytic rate calculated from experiments of 1 hour's duration, which give similar figures to the earlier experiments of 2 hours' duration. This is also seen from Table XI where two experiments of 1 hour's duration are recorded.

Table XI. *Glucolytic rates in experiments of one hour's duration only.*

All fluids contained 0·2 % glucose.

Gas	Medium	mg. lactic acid per g. tissue per hour Exp. 1	 Exp. 2
N_2	Ringer	48	64
N_2	„ $+0{\cdot}1M$ KCl	18	25
O_2	Ringer	23	31
O_2	„ $+0{\cdot}1M$ KCl	47	75

Since very high glucolysis is observed in O_2 and KCl, and it has previously been suggested that the lactic acid formed in N_2 is removed in O_2 [Holmes, 1930; Krebs, 1931], it seemed of importance to see if the aerobic removal of lactic acid were inhibited by KCl. Accordingly the rate of disappearance of 0·05 % lithium lactate was measured both in presence and absence of potassium. The figures recorded (Table XII) show that the aerobic removal under conditions similar to those of the former experiments is of a very low order but is not markedly affected by potassium.

Table XII. *Oxidative removal of lactic acid in presence and absence of 0·1* M *potassium chloride.*

Exp. No.	Duration hours	mg. lactic acid removed per g. dry tissue Ringer alone	 Ringer $+0{\cdot}1M$ KCl
1	2·5	17	21
2	2·0	25	32
3	2·0	36	36

It was attempted to ascertain if any intermediaries accumulated in the presence of potassium salts both in O_2 and N_2. The trichloroacetic acid extract when tested for pyruvic acid by the nitroprusside reaction was negative, nor did potassium influence the bisulphite-binding capacity of the extract, which was in any case small.

One further point has been investigated. Dodds and Pope [1933] showed that 2 : 4-dinitro-*o*-cresol increased the metabolic rate of mice and Dodds and Greville [1934] have shown that this substance increases both respiration and aerobic glucolysis in tumour tissue. They did not study the effect on anaerobic glucolysis. It would seem possible that potassium salts might also affect in some way the respiration of brain slices. An earlier preliminary experiment in the Barcroft manometer and in phosphate buffer had suggested that this was not the case, and the point was not pursued, except to show that the rate of lactic acid oxidation was not affected in the presence of potassium, at any rate in bicarbonate buffer. Some manometric experiments have now been made using the Warburg indirect method and simultaneous measurements of oxygen consumption and aerobic glucolysis and also anaerobic glucolysis have been carried out. The results shown in Table XIII amply bear out our results obtained by the chemical method, namely that anaerobic glucolysis is inhibited, while aerobic glucolysis is markedly increased, by 0·1M KCl. Further the very interesting fact emerges that the respiration is also increased by values which range from 50 to 200 % of the normal respiration. Except in Exp. 1, there is no apparent correlation between the increase in respiration and the increase in aerobic glucolysis, which might suggest that in the presence of potassium there is a general stimulation of the aerobic metabolism of the cell and that this increased aerobic glucolysis is one method of manifestation of this phenomenon. The significance of these results is discussed again later. It will be noticed (Table XIII) that the

Table XIII. *The effect of* M/*10 potassium chloride on the respiration and glucolysis of brain slices.*

Exp.	(I) Ringer + M/10 KCl					(II) Ringer alone						
No.	Q_{O_2}	Q_{S,CO_2}	$Q_L^{O_2}$	$Q_L^{O_2}$*	$Q_L^{N_2}$	Q_{O_2}	Q_{S,CO_2}	$Q_L^{O_2}$	$Q_L^{O_2}$*	$Q_L^{N_2}$	MQ	Remarks
1	19·0	30·5	11·5	—	4·5	6·5	6·5	0	—	15·5	2·4	
2	10·5	21·0	10·5	—	—	4·0	4·5	0·5	—	—	—	
3	11·5	22·5	11·0	—	—	7·5	8·5	1·0	—	—	—	
4†	16·5	33·5	17·0	15·5	→	11·0	15·5	4·5	3·0	—	—	In this exp. the tissues were transferred from (I) to (II) after one hour
5†	24·5	42·0	17·5	15·5	→	13·0	19·0	6·0	4·5	—	→	Ditto, but after 2nd hour tissue returned to medium (I)
		(1st hour)					(2nd hour)					
	19·0	31·0	12·0	10·5	—	—	—	—	—	—	—	
		(3rd hour)										
6	21·5	42·0	20·5	18·5	→	—	—	—	4·5	—	→	Exp. as exp. 5
		(1st hour)					(2nd hour)					
	—	—	—	9·5	—	—	—	—	—	—	—	
		(3rd hour)										

* Indicates that the results in this column were obtained by the simplified method. All other results by the indirect method (*i.e.* $Q_L^{O_2} = Q_{S,CO_2} - Q_{O_2}$).

† Indicates 0·2M bicarbonate. All others in 0·25M bicarbonate.

→ Indicates transfer of tissue from one medium to another.

aerobic glucolysis in the absence of potassium chloride is practically zero, whereas our chemical estimations of $Q_L^{O_2}$ vary from 2·5 to 5·0. Such figures correspond to 10–20 mg. lactic acid per g. of dry weight. This discrepancy is almost certainly due to the fact that some lactic acid remains even in the washed tissue slices and of course is estimated in the chemical determinations, whereas the manometric method is unaffected by initial lactic acid in the tissue.

These results also show that the addition of M/10 KCl inhibits the "Pasteur reaction" in brain tissue, if we accept as a criterion of this inhibition a rise in aerobic glucolysis without any fall in respiration (see Dickens [1934] for further discussion on this point). Moreover, this inhibition is largely reversible as is seen from Exps. 4, 5 and 6 in Table XIII. In each of these the same tissue slices were used throughout. After a period of one hour in the Warburg pots containing Ringer and KCl, the slices were transferred to a second pair of pots containing Ringer without excess KCl, after, of course, washing in normal Ringer. In Exps. 5 and 6, a second transfer back to Ringer and KCl was effected. The manometric results obtained during these periods in different media show that the high aerobic glucolysis and oxygen uptake, characteristic of the slices in KCl, fall to lower and normal values when KCl is no longer in the medium and return again to the high values when $M/10$ KCl is again present. The effect of KCl on the aerobic process is therefore reversible.

One point should be mentioned in connection with these experiments. In Exp. 6 only pots containing a small volume of fluid were employed after the first transfer, so that O_2 uptake was not determined. However, a value for aerobic glucolysis ($Q_L^{O_2}$) can be obtained since the volume used was only 1·5 ml. and it is assumed that none of the CO_2 liberated was retained. How far this assumption is warrantable is shown by comparing the value of $Q_L^{O_2}$ calculated by this simple method with the values obtained by the complete indirect method with pots containing both large (5·5 ml.) and small (1·5 ml.) fluid volumes as shown in Exps. 4, 5 and 6. The values obtained with the simplified method are indicated with an asterisk.

Discussion.

The main result of these experiments, namely, that the anaerobic glucolysis is markedly decreased by the addition of K salts while the aerobic glucolysis is raised to be several times as large as the anaerobic, is hard to explain. It would seem possible that the potassium would merely inhibit the Pasteur reaction and so raise the aerobic glucolysis. At the same time this concentration of potassium might also inhibit the anaerobic glucolytic system. The explanation would only be feasible if the aerobic glucolysis were raised and the anaerobic glucolysis lowered until they attained one and the same level. It cannot possibly be put forward, however, in view of the fact that so far from attaining the same level the aerobic glucolysis is several times as large as the anaerobic in the presence of added potassium.

A possible explanation is that the large formation of lactic acid in the presence of KCl is due to a different system coming into play from that occurring in normal anaerobic glucolysis. One could assume that owing to the presence of potassium some substance in the normal glucolytic chain accumulates slightly, in that its further reaction is inhibited. If, however, this substance could be oxidised directly to lactic acid, then formation of lactic acid would occur in oxygen. Lactic aldehyde is a substance which on oxidation would give lactic acid; it would be interesting to ascertain the effect of this substance on brain slices in view of the fact that it is known to disappear in liver "brei" as does methylglyoxal [Neuberg, 1913]. An argument which militates against a second method of lactic acid formation occurring in O_2 and KCl is that this glucolytic system like the normal anaerobic mechanism is inhibited markedly by glyceraldehyde. However, glyceraldehyde may act at some earlier point common to both mechanisms.

Another possibility, which Dr H. A. Krebs has put before us, is that KCl acts by injury to the slices, thus damaging the glucolytic mechanism, and that this damage is greater in N_2 than in O_2 where only the Pasteur effect is eliminated. This would explain the non-recovery of high glucolysis in N_2 on placing slices again in normal Ringer after they had been incubated in N_2 and KCl for 1 hour, and also the failure to obtain high glucolysis on bubbling O_2 through the medium in which slices were suspended which had been previously incubated anaerobically in the presence of potassium. However, since normally glucolysis occurs to such a high extent under anaerobic conditions, and also since anaerobiosis alone does not seem to kill the brain cells quickly in the presence of glucose, it is difficult to see how the mere presence of O_2 should impede the damage to the cells by potassium when KCl in O_2 does not cause this damage.

It would seem probable that the damage is connected with the inhibition of glucolysis by potassium. Under aerobic conditions there is still a mechanism of lactic acid formation, and this, or oxidation of other substances, may give the cells a necessary metabolic reaction. In N_2, however, glucolysis can no longer proceed, and so the cells are injured irreversibly, perhaps as they have no longer a means of obtaining the energy necessary for life. In this regard it is interesting that Dickens and Greville [1933] have shown that incubating brain slices at 38° for as short a time as 20 mins. anaerobically and without glucose destroys all but 12 % of their power of glucolysis when glucose is added. From these considerations the irreversibility of the effect of addition of KCl in N_2 does not seem contrary to the above theory of two different glucolytic mechanisms, in nitrogen and in oxygen.

From the results of our experiments (Table XIII) in which both respiration and glucolysis were determined, it may be said that KCl definitely inhibits the Pasteur reaction (and further that this inhibition is reversible) provided that the Pasteur reaction is regarded strictly as the inhibition of lactic acid formation by oxygen. It must be emphasised, however, that the mechanism of formation of lactic acid in oxygen when KCl is added may not necessarily be the same as that occurring normally in nitrogen; in fact this appears unlikely in view of the inhibitory effect of potassium chloride on anaerobic glucolysis. Secondly, as well as increasing aerobic glucolysis, the addition of potassium markedly increases the respiration. The significance of this is uncertain; it may be connected with general stimulation of metabolism or perhaps oxidation of some intermediary to lactic acid. It is possibly due to the increasing concentration of lactic acid, but we have shown that the rate of removal of lactic acid is not affected. There is apparently no correlation between increase in oxygen uptake and aerobic glucolysis, though it appears that a relation exists between the total oxygen uptake and the aerobic glucolysis in experiments in which $M/10$ KCl was added. Further the question asked by Dickens [1934], as to whether aerobic glucolysis can be increased in tissues where carbohydrate oxidation is not diminished, can now be answered. These conditions are satisfied in brain slices glucolysing aerobically in the presence of KCl, since presumably here the high respiration is due to an oxidation of the carbohydrate type [see Dickens and Sîmer, 1930]. Thus we can say that KCl does not behave in the same way as Dickens [1934] has suggested for phenylhydrazine, since this substance does not inhibit anaerobic glycolysis.

As regards the original purpose of these experiments, that is to see if oxygen interferes with lactic acid production by removing some reactant in the glucolytic chain, we have not succeeded in demonstrating any positive evidence for this theory by addition of possible substances and so are thrown back on either the explanation of Meyerhof or that of Lipmann as to the mechanism of the Pasteur effect.

From our results, however, the former explanation, that is that lactic acid formed as in N_2 is merely removed by the oxygen, becomes unlikely, at any rate in brain, since if this were the case it would be hard to explain how potassium so markedly increases the aerobic glucolysis when it apparently does not interfere with the removal of lactic acid from brain slices under aerobic conditions. It is possible, however, in view of the increased O_2 uptake, that the general metabolism might be so stimulated as to increase the rate of lactic acid formation in the presence of O_2 and KCl. However, it seems unlikely that increased O_2 uptake would be associated with increased lactic acid production unless the lactic acid production were caused by some direct oxidation. This is, in fact, the same as postulating another mechanism of lactic acid formation coming into play in O_2 and KCl. Another argument against the view that lactic acid is formed as under anaerobic conditions but removed in O_2 is the slow rate of removal of added lactate in brain (Ashford and Holmes [1931] and our more recent observations on brain slices) compared with the high rate of lactic acid formation by similar tissue under anaerobic conditions.

From this we may conclude that the theory of Lipmann is not, as the others are, at variance with these observations. The mechanism of the action of potassium salts is at present completely obscure, and the existence of another mechanism for glucolysis occurring in tissues in O_2 and KCl is still a possibility. An even more disturbing possibility is that under artificial conditions a cell may actually develop a new and different mechanism. If this were so it would throw into doubt much work done on excised tissues and extracts. At present it is simpler to postulate that there are different mechanisms for lactic acid production in oxygen and in nitrogen and that the former is activated by potassium and the latter inhibited.

SUMMARY.

1. The addition of potassium α-glycerophosphate to glucolysing brain slices was found to increase the aerobic and inhibit the anaerobic glucolysis, so that the aerobic glucolysis became several times greater than the anaerobic. This effect is not shown by the sodium salt.

2. Potassium chloride, but not sodium chloride, was found to have the same effect in $0{\cdot}1M$ concentration, the aerobic glucolysis assuming values from 3 to 6 times as large as the much diminished anaerobic glucolysis. The phenomenon is due entirely to the potassium ion.

3. The effect of varying concentrations of potassium chloride on both aerobic and anaerobic glucolysis in brain slices was studied.

4. Potassium does not affect the oxidative removal of lactic acid.

5. *dl*-Glyceraldehyde was shown to inhibit the raised aerobic glucolysis occurring in the presence of $M/10$ KCl.

6. The addition of pyruvate did not increase the aerobic glucolysis or remove the inhibitory effect of potassium on the anaerobic phase.

7. The oxygen uptake was shown to be increased in the presence of $M/10$ KCl.

8. The reversibility of these effects has been investigated. The inhibition of anaerobic glucolysis by KCl in N_2 was shown to be irreversible, whereas the increase in aerobic glucolysis and oxygen uptake by addition of KCl during aerobiosis is reversible.

9. The bearing of these results on the significance of glucolysis and on the Pasteur effect is discussed.

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REFERENCES.

Ashford (1933). *Biochem. J.* **27**, 903.
—— (1934). *Biochem. J.* **28**, 2229.
—— and Holmes (1929). *Biochem. J.* **23**, 743.
—— —— (1931). *Biochem. J.* **25**, 2028.
Beebe (1904). *Amer. J. Physiol.* **12**, 167.
Bumm, Appel and Couceiro (1933). *Z. physiol. Chem.* **220**, 186.
Clowes and Frisbie (1905). *Amer. J. Physiol.* **14**, 173.
Dickens (1934). *Biochem. J.* **28**, 537.
—— and Greville (1933). *Biochem. J.* **27**, 1134.
—— and Simer (1930). *Biochem. J.* **24**, 905.
Dodds and Greville (1934). *Lancet*, i, 398.
—— and Pope (1933). *Lancet*, ii, 352.
Embden, Deuticke and Kraft (1933). *Klin. Woch.* **12**, 213.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Holmes (1930). *Biochem. J.* **24**, 914.
Krebs (1931). *Biochem. Z.* **234**, 278.
Lasnitzki (1933). *Biochem. Z.* **264**, 285.
Lipmann (1933). *Biochem. Z.* **265**, 133.
Mendel (1929). *Klin. Woch.* **8**, 169.
—— Bauch and Strelitz (1931). *Klin. Woch.* **10**, 118.
Meyerhof and Kiessling (1933). *Biochem. Z.* **264**, 40.
—— Lohmann and Meier (1925). *Biochem. Z.* **158**, 459.
Neuberg (1913). *Biochem. Z.* **49**, 502.

XXII. THE LIVER AND FAT METABOLISM.

By FRANCIS XAVIER AYLWARD, HAROLD JOHN CHANNON
AND HARRY WILKINSON.

From the Department of Biochemistry, The University, Liverpool.

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The outstanding evidence of the importance of the liver in fat metabolism is the intense accumulation of fat which occurs in this organ in widely varying experimental and pathological conditions. Known observations however do not at present provide any adequate reason for this fat accumulation. The desaturation hypothesis of Leathes [1925], based on the work of Leathes and Meyer-Wedell [1909] and of Hartley [1909], presents an attractive suggestion of the reasons why fat may be transported to the liver, for apart from the desaturation of the fatty acids themselves it serves to connect the metabolism of glycerides with that of liver phosphatides. In the last three years work in this laboratory has been directed towards an attempt to obtain further evidence on the function of the liver in fat metabolism, and part of this work has referred in particular to the desaturation theory. The results of these investigations [Channon *et al.* 1934, 1, 2,] together with those of other workers discussed in the former paper, leave us reluctant to accept that part of the hypothesis which is concerned with desaturation until more positive evidence in its favour is acquired. On the other hand, although the investigations of the French school and of many other workers seem to suggest that the phosphatides of tissues and in particular those of the liver may be essential constituents of living cells—"l'élément constant" of Terroine [1919]—yet close consideration makes it not unlikely that liver phosphatides may possess an active metabolic function, for they vary in amount in certain conditions. The fact that the percentage of liver phosphatide usually falls when fat infiltration occurs is an example of such a variation. Further, the extensive work of Best and collaborators [*cf.* Best, 1934] shows the remarkable effect of small amounts of choline in prevention and cure of fatty livers produced by various experimental conditions. Although the effect of choline may be exercised in many ways, one possible means is through the metabolism of choline-containing phosphatides.

The work described in this paper was carried out with a view to obtaining further evidence on the rôle of the liver in fat metabolism. The frequent accumulation of fat in the liver suggested that some changes should be detectable after a meal containing fat. Little definite information on this subject is available, although Artom [1933], who provides a summary of the subject, has obtained results which will be discussed later. It appeared possible that the lack of advance in this field might be due to several reasons. Firstly, any small changes in the amount of the liver lipoids after fat absorption may have been generally masked by variation of the individual animals employed—a fact which appears to have been insufficiently appreciated; for this reason we have used in this work the pooled livers of groups of animals. Secondly, analyses have generally been made at relatively long time intervals and estimations of the total ether-soluble hydrolysis products only have usually been carried out. The former procedure might well fail to reveal a dynamic function of the liver in fat metabolism, while the latter would mask slight changes in the individual lipoid constituents. Thirdly, the recent work of Best and his colleagues suggests that some at least of the negative results may have been due to the presence of choline in the diets employed. If choline is a normal physiological agent for the control of the amount of fat in the liver, the provision to animals of a meal containing fat, but free from choline, would be more likely to result in a transient rise in liver fat. Because work is being carried out in this laboratory on the "cholesterol" fatty liver, it was decided to include cholesterol in the diet with a view to obtaining further evidence on this problem also.

In order that the results might be of greater value in attempting to assess the normal processes occurring in liver following fat absorption, it was felt preferable to administer a diet containing all the primary food constituents, protein, fat and carbohydrate, in place of the exclusively fatty diet which has often been employed in researches of this type, because the production of fatty livers by dietary methods seems to depend on a number of factors concerned with the proportion of all three constituents in the diet, apart from the effect of choline. The general plan of the experiment therefore was to feed a large number of animals on a choline-free diet containing 40 % of fat and 2 % of cholesterol. A similar number received the same diet with the addition of choline chloride. From each series eight animals at a time were killed at intervals up to 13 hours and the amounts of phosphatide, glyceride, cholesterol and cholesteryl ester present in the livers determined.

EXPERIMENTAL.

Eighty rats, which had been maintained on the laboratory stock diet for a considerable period, were divided into ten groups, each of eight animals (3 bucks and 5 does) to give as even weight distribution as possible. Two of these groups were used as controls. Of the remainder, four groups received diet C and four groups diet D. The groups in each series received a corresponding serial number depending on the time at which the animals were killed after receiving their particular diet.

All the animals were liberally fed on the normal stock diet for the last time at 10.0 a.m. on the day before the experiment. At 8.0 p.m. the remainder of the food was removed from the cages and the experiment began on the following day at 9.30 a.m., 13½ hours after food was last available to the animals. At this time weighed amounts of the diets C and D were placed in small dishes in all the cages save those of the two control groups. At 10.30 a.m. the dishes were removed, after carefully adding any food which had been scattered about the cages. The animals thus had access to the diets for 1 hour only. One group from each of the two series was killed at 4, 7, 10 and 13 hours after the provision of the food (9.30 a.m.). The sixteen animals used as the control group were killed at 9.30 a.m., they themselves thus having had no food for 13½ hours.

The composition of diet C was: fat 40, caseinogen 20, glucose 40, cholesterol 2, water 8 parts; diet D was the same as diet C with the addition of choline chloride, 2 parts.

The fat used in the diets was freshly prepared dripping obtained from beef fat by melting and filtration.

The caseinogen used was Glaxo "fat-extracted casein," which as received contained less than 0·2 % "fat." Before use this was extracted four times with boiling industrial alcohol and then four times with ether in order to remove any trace of phosphatide present. By biological assay on rabbit intestine, after hydrolysis followed by acetylation, diet C was found to contain less than 1 mg. choline per 100 g.

The method of mixing the diets was to make the sugar into a paste with water in the case of diet C and with choline chloride solution in the case of diet D. The caseinogen was then added and well mixed. The cholesterol was added to the melted fat and the mixture heated with stirring until the cholesterol had dissolved. It was then added to the sugar-caseinogen mixture. It is to be emphasised that every care was taken by prolonged kneading to ensure that the diet was uniform.

Extraction of the tissues and analysis of the extracts.

Each rat was weighed and then killed by a blow on the back of the head. The livers were removed and weighed, those from each group being pooled as one sample. The alimentary tracts were dissected out, freed from adherent fat as far as possible and the portion from the oesophagus to just above the caecum removed for analysis. The procedure adopted for the extraction of the livers was one which would enable the dry weight of the livers and that of the total ethereal extract to be obtained [Channon and Wilkinson, 1934]. The analyses of the ethereal extracts were carried out as described in that paper and included determinations of the total fatty acids and unsaponifiable matter obtained by alkaline hydrolysis, ether-soluble phosphorus and free and total cholesterol, together with the iodine values of the total fatty acids.

The amount of fat ingested and the amount absorbed were also determined. The fat intake was calculated from the difference between the weight of food administered and that remaining uneaten in the cages. From this figure and that obtained for the fat remaining in the tracts at the time of killing, the amount of fat absorbed could be calculated. The alimentary tracts from each group were therefore heated with 300 ml. 5 % potassium hydroxide for 12 hours. The resulting dark brown solution was acidified in a separating funnel and extracted with ether. Considerable difficulty was encountered because of emulsion formation, which could usually be overcome by the use of alcohol. The extraction difficulties were, however, such as to make it possible that the results obtained were only approximately correct. The combined ethereal extracts were washed with water five times and the "fat" obtained by removal of the solvent *in vacuo*. It was dissolved in light petroleum and allowed to stand overnight, when the purified "fat" was obtained by filtering the light petroleum solutions and removing the solvent *in vacuo*. The resulting material consisted of a mixture of fatty acids and unsaponifiable matter, together with some unhydrolysed glyceride. It was not further hydrolysed to convert it entirely into its constituent fatty acids and unsaponifiable matter, because the figure obtained would not differ sufficiently greatly from the weight of the extract already obtained to make any material difference in the interpretation of the results. From the weight, therefore, one half of the corresponding figure from the tracts of the 16 control animals was subtracted. The fat absorbed was then obtained by subtraction of the resulting figure from that of the fat eaten.

RESULTS.

The detailed figures for the water contents of the livers will not be recorded, for no significant variation was observed, the extreme values for all the groups being 69·3 and 71·6 %; nor is it necessary to present the results of the analyses of the ethereal extracts of the livers in detail. The composition of the liver lipoids as set out in Table I has accordingly been calculated. The ether-soluble phosphorus is assumed to be present as a dioleyllecithin (P 3·86 %, fatty acids 70·4 %). The cholesteryl ester is calculated as the oleate. $\left(\text{Total cholesterol determined on the unsaponifiable fraction } minus \text{ the free cholesterol, } \times\frac{650}{386}.\right)$ The glyceride fatty acids have been deduced by subtracting from the total fatty acids those present in the phosphatide (70·4 %) and those present as cholesteryl ester $\left(\text{cholesteryl oleate } \times\frac{282}{650}\right)$. This difference multiplied by 1·045 yields the figure for glyceride as triolein.

The only other point which need be mentioned is that the weights of liver for groups C 1, C 2, C 3 and C 4 were 53·0, 54·6, 56·0 and 51·2 g. respectively, while the corresponding figures from the D series were 57·3, 52·0, 50·2 and 47·5 g.

Table I. *Composition of the liver lipoids (g./100 g. liver).*

Group	Hours after provision of food	Phosphatide	Glyceride	Free cholesterol	Cholesteryl oleate	I.V. of total fatty acids
Control	—	3·51	0·943	0·261	0·00	122·6
C 1	4	3·43	0·888	0·276	0·00	123·5
C 2	7	2·65	1·411	0·257	0·032	110·8
C 3	10	3·15	1·863	0·269	0·113	95·5
C 4	13	3·45	1·361	0·258	0·202	—
D 1	4	3·28	0·861	0·261	0·002	119·9
D 2	7	3·19	1·226	0·283	0·037	112·3
D 3	10	3·35	1·455	0·269	0·106	106·4
D 4	13	3·40	1·105	0·264	0·173	—

DISCUSSION.

One preliminary observation to be made concerns the variation to be expected in the amounts of the different lipoid constituents of the livers of individual animals maintained on a stock diet. Much evidence is available to show that when fat infiltration into the liver occurs, its intensity in individual animals may be strikingly dissimilar. This variation is so great that even batches of pooled livers of groups of several rats, maintained under the same nutritive conditions, may yield analytical results definitely differing one from the other. On the other hand the variation in the amounts of the different lipoid constituents in the livers of rats maintained for considerable periods on a normal constant stock diet and kept without food for twelve hours is small. In these conditions the phosphatide and the free cholesterol show very little variation, while cholesteryl esters are virtually absent. The greatest variation likely to be met with is in that of the neutral fat fraction. In the work under discussion each group consisted of eight animals in the belief that in the conditions of these experiments this number would be sufficiently great to make for reliability in the interpretation of any changes which might occur. This point is mentioned because some authors have used individual animals in such studies and because possible variability must be borne in mind in the interpretation of the results.

The variations in the individual constituents will now be discussed in turn and it will be convenient if these be presented together in a series of curves, because certain reciprocal relationships are thereby made apparent. In Fig. 1 the sequence of changes occurring in the amounts of phosphatide and glyceride and in the iodine values of the total fatty acids are set out.

(*a*) *The phosphatide fraction.* Fig. 1 shows that the phosphatide percentage in the livers of animals which did not receive choline decreases slowly for a period of 4 hours and then falls more sharply to a minimum value, 24·5 % below the control value, at the seventh hour, to rise again to normal after 13 hours. In the D series, however, in which the animals received choline, the corresponding decrease in the phosphatide percentage, 9 %, is not nearly as pronounced. This finding implies one of two things. Either choline has prevented the partial disappearance of liver phosphatide, which according to these results normally occurs after a fatty meal, or alternatively its presence in the diet has enabled synthesis of phosphatide to occur and this newly synthesised phosphatide has replaced that which has disappeared. The latter explanation seems the more probable.

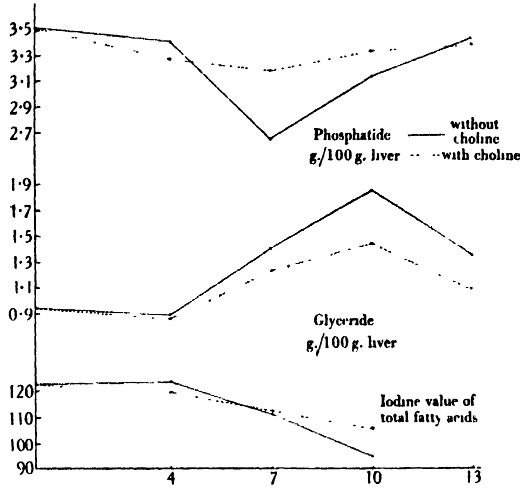

Fig. 1.

Hence, after a meal containing fat and cholesterol, the liver phosphatide falls until the seventh hour and this fall is checked in part by choline administration. It seems possible that this observation may prove a step forward in the solution of the problem as to how choline prevents and cures certain types of fatty liver. It is well known that the phosphatide content of fatty livers is usually markedly decreased and further that such livers after "cure" by choline contain a normal amount of phosphatide. The results under discussion show that a normal fall in phosphatide occurs after the ingestion for 1 hour of what is not a very unphysiological diet for a twelve-hour experiment in the sense that it contained carbohydrate, fat and protein in the ratio of 2 : 2 : 1, and that this fall may be prevented in part by the administration of choline. The unbalanced diets of high fat content and containing little or no choline which have been used to produce fatty livers have been employed for relatively considerable periods (21 days) and from the results under discussion experiments of such a type might be expected to yield fatty livers, for fat absorption in these conditions would be a more or less continuous process and any depletion of liver phosphatide might be persistent. This fall in liver phosphatide may be related to the rise in blood lecithin which has often been reported to occur after a fatty meal at a similar time.

(*b*) *The glyceride fraction.* It appears to be legitimate to make three deductions from the curves in Fig. 1. If the curve for the livers of the animals which did not receive choline be considered, it is seen that no change occurs in the glyceride content of the liver for four hours. In the next six hours, however, a rapid increase occurs and this is steadily maintained to the tenth hour, when a rapid return towards normal occurs in the next three hours. Although many conflicting results occur in the literature, the general outcome of blood fat studies is that a maximum is reached about the seventh hour, followed by a return to normal at the twelfth hour. The curves thus bring out the first point, namely that the amount of liver glycerides varies in a manner somewhat similar to that of blood glycerides after a fatty meal.

The second consideration refers to the relationships between the amounts of the glyceride and phosphatide fractions. Both remain approximately constant until the fourth hour. At this point the phosphatide fraction begins rapidly to decrease while the glyceride fraction immediately increases at an equal rate. At 7 hours the phosphatide is at its minimum value and the glyceride is still increasing, to reach a maximum value at the tenth hour and to fall steadily without completely reaching the normal figure after 13 hours. There thus seems to be a reciprocal relationship between the amounts of phosphatide and glyceride in the liver after a fatty meal.

The third point worthy of mention concerns the effect of choline. Best *et al.* [1934] deduced that the primary effect of choline was on the glyceride fraction of the liver lipoids. In Fig. 1 the curve for the glyceride of the livers of the animals which received choline is at a lower level than that of those which received the choline-free diet. Although the differences are not profound, they exist at every hour at which analyses were made. It seems reasonable therefore to suggest, according to these results, that the effect of choline is observable even in the hours immediately following a fatty meal.

(*c*) *The iodine values of the total fatty acids.* No significant change in the iodine values of the fatty acids of either group occurs in the first four hours. At this point, however, a progressive fall appears, which is maintained to the tenth hour. Further, in the case of group D, which received choline, the value at this point, 106·4, is considerably higher than that of C, 95·5. Unfortunately a breakage prevented the iodine values of groups C 4 and D 4 from being determined. These observations seem in complete accord with the discussion under (*a*) and (*b*). The decrease in phosphatides results in a loss to the liver of their constituent highly unsaturated acids. At the same time as this occurs, the relatively saturated acids of the food fat, of iodine value 40, are entering the liver, with a resultant fall in iodine value. This fall is not nearly as pronounced at the tenth hour in group D, because choline has definitely, though in part only, prevented the increase. Thus while group C at this stage contains 1·863 g. of glyceride per 100 g. of liver, the choline group D contains only 1·455 g. These findings are in accord with the previous work of many authors, who have studied the production of fatty livers by various means, in showing that the iodine value of the liver fatty acids decreases progressively with fat infiltration. Imrie [1914] showed that the degree of fat infiltration could in fact be calculated mathematically from a consideration of the iodine values of the total liver acids, although in our experience such a relationship does not hold strictly. The present results are, however, more particularly interesting in that in these experiments there is no obvious increase in the amount of the total liver fatty acids, the maximum figure reached being 4·049 g. per 100 g. of tissue in group C 3, an increase of only 0·678 g. over the initial value. Calculations which need not be described here lead us to the view that an elaboration of these experiments may throw further light on the desaturation theory and to this problem we are giving attention.

The results under (*a*), (*b*) and (*c*) therefore seem to yield a picture of the normal sequence of events in the liver after ingestion of a meal containing fat and cholesterol, very similar to that which occurs when fatty livers are produced, *viz.* a fall in liver phosphatide, an increase in glyceride and a consequent lowering of the iodine value of the total fatty acids. They suggest a physiological explanation of the fatty liver produced by dietary methods and its prevention and cure by choline administration. For a number of reasons, however, we feel that caution should be exercised in their interpretation. The constancy in the amount of the liver phosphatides has been emphasised by the work of Terroine and Bélin [1929] and of Sinclair [1929] and a fixed impression of this constancy has arisen, whilst hitherto experimental evidence of a change implying a quantitative participation of the liver phosphatides in normal fat metabolism has been, in general, lacking. Little evidence of the latter type is available save that of Artom [1933]. This investigator found that the amount of phosphatide in the livers of dogs starved for periods up to 36 hours was very constant. He then administered to other dogs, starved for similar periods, horse fat to the extent of 20–35 g. per kg. of body weight. Using single animals—a procedure which was quite justified by his findings on his control animals—he found a steady increase in liver phosphatide to the sixth and seventh hours, followed by a return to normal at the tenth hour. His maximum phosphatide increase, 36 %, was obtained at the very time when our maximum decrease, 25 %, occurred. The contradiction of these results is striking and difficult to explain, although many factors may be involved. The amounts of fat ingested by his animals were very high and no other food was given. Further, on basal metabolic considerations the preliminary period of starvation, 12 to 36 hours, was relatively insignificant for his dogs, compared with the corresponding period of 13½ hours which preceded our experiment with rats. The absence of choline from our diet, which included 40 % of carbohydrate and 20 % of protein in addition to 2 % of cholesterol, may be a further cause of the discrepancy.

That the conditions governing the amount of phosphatide in the liver are many was illustrated by a further experiment on rats, in which twelve animals were starved for 17¼ hours, after which six were killed and six were given diet C for a period of 1 hour. Analysis of the livers of the control group and of the fed animals 6½ hours after feeding gave the following results, the control figures being recorded first: total fatty acids 3·17, 4·22 %; I.V. of total fatty acids 101, 74; total cholesterol 266, 360 mg. per 100 g.: ether-soluble phosphorus 122·6, 119·4 mg. per 100 g. Thus in this experiment the changes in the amounts of total fatty acids and cholesterol and in the iodine values of the fatty acids were very similar to those recorded in the previous experiment. On the other hand there was no significant change in the amount of phosphatide. The increase in the amounts both of fatty acids and cholesterol suggest in comparison with the results of the previous experiment that the phosphatide decrease may have occurred earlier, and this different finding may be correlated perhaps with an increased time of starvation. In any case these results also differ from those of Artom and indicate the complex nature of the problem.

For these reasons it is preferable to defer further consideration of the general significance of the findings recorded in this paper until more results are available. We are therefore extending the observations to similar studies on diets containing fat, but free from choline, and with the omission of cholesterol, which may easily be a complicating factor, and we are undertaking a comprehensive study of the blood lipoids at the same time. If the suggestive results concerning a mobilisation of phosphatide from the liver after fat absorption be confirmed, they can then be related to the rise in the amount of blood phosphatide, and the effect of choline administration on the other blood fat constituents can also be studied.

It is to be mentioned also that the amount of fat absorbed by the different groups did not increase regularly with time. Thus groups C 1 to C 4 absorbed 4·90, 4·34, 2·62 and 15·17 g. respectively, while groups D 1 to D 4 absorbed 3·23, 2·90, 7·29 and 6·74 g. The irregularity of these amounts may appear surprising in groups of equal numbers of animals starved for a similar period. We have observed such an irregularity in the amount of fat absorbed on other occasions and we have no explanation to offer for it; it is also possible that some of the results may be connected with this varying degree of absorption. It is of interest also to attempt to assess the amount of choline absorbed in groups D 1 to D 4. The total choline chloride intake of groups D 1 to D 4 was 0·78, 0·67, 0·68, 0·58 g. respectively. Each animal in all the groups thus ingested about 60 mg. of choline and presumably this amount was absorbed.

(*d*) *Cholesterol.* Table I shows that no significant changes occur in the amounts of free cholesterol in the livers of either groups, the extreme values being 257 to 283 mg. per 100 g. of liver. On the other hand striking increases in the amount of cholesteryl ester took place in the livers of both groups of animals. These are illustrated in Fig. 2.

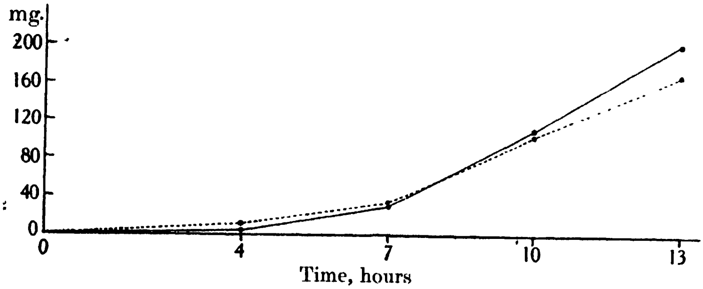

Fig. 2. Cholesteryl ester mg./100 g. liver.
—— Without choline. ------ With choline.

From Fig. 2 and Table I a number of deductions may be made. In the first place it is clear that within four hours of taking a meal containing cholesterol, cholesterol appears in increased amount in the liver of the rat. The whole of the increase in liver cholesterol throughout this experiment is due to an increase in cholesteryl esters (Table I). This result therefore illustrates the initial stage of the production of the "cholesterol" fatty liver, which is characterised by the appearance in the liver of excessive amounts of cholesteryl esters with a large but varying increase in the amount of neutral fat [Okey, 1933, 1, 2; Chanutin and Ludewig, 1933; Best *et al.* 1934; Channon and Wilkinson, 1934]. The latter authors discuss the conditions which influence the degree to which each of these two constituents is increased, for it is clearly an important point to determine why cholesterol appears in the liver exclusively in ester form, with a greater or lesser rise in the glyceride content. The intensity with which this process occurs when cholesterol is fed seems from the present results to depend on the relative inability of the animal to metabolise the substance sufficiently rapidly. After a meal containing it accumulation in the liver begins at once and hence the intensity of the final result of prolonged cholesterol feeding. Determinations of the cholesterol left in the alimentary tracts of the control group and of groups D 3 and D 4 showed that the latter had absorbed 489 and 425 mg. respectively in 9 and 11½ hours. Calculation from the liver weights and the percentages recorded in Table I shows that at these times the amounts of cholesteryl oleate present in the livers of groups D 3 and D 4 were 74 and 94·5 mg. respectively, which correspond to 43 and 58 mg. of cholesterol. Clearly therefore the increase in liver cholesterol represents only part of the cholesterol absorbed.

One further observation which may be made is that, in the twelve hours following a fatty meal containing cholesterol, choline administration has had no marked effect in preventing cholesterol accumulation in the liver, a result which seems concordant with those of Best *et al.* [1934] and Channon and Wilkinson [1934]. It suggests that whereas the amounts of phosphatide and glyceride in the liver are mutually interdependent, the amount of cholesteryl ester is not directly involved in the same mechanism of control.

SUMMARY.

1. A study has been made of the variations in the liver lipoids of rats (*a*) receiving a meal of a diet consisting of sucrose 40, caseinogen 20, fat 40 and cholesterol 2 parts, but free from choline, and (*b*) receiving a meal as under (*a*) with the addition of choline chloride, 2 parts. The changes in the liver lipoids were studied over a period of 13 hours.

2. The significant findings were as follows:

(*a*) *The phosphatide fraction.* For a period of four hours little change occurred in the percentage of phosphatide. There was then a fall to a minimum value at the seventh hour, followed by a return to normal at 13 hours. In the corresponding animals which received choline these phosphatide decreases were less than one-third as great. The evidence produced thus indicated that choline prevented at least in part the fall in the amount of liver phosphatide which took place in its absence.

(*b*) *The glyceride fraction.* The livers in both series of animals showed no change in neutral fat content for about four hours. There was then a rapid increase to the tenth hour, followed by a return towards normal at the end of thirteen hours. The effect of choline appears consistently in these curves, for the glyceride percentages in the livers of animals which received choline were uniformly lower than that of the control series.

(*c*) *The iodine values of the liver fatty acids.* The iodine values of the total fatty acids decreased in both groups during the first ten hours. The results under 2 (*a*), 2 (*b*) and 2 (*d*) are discussed in detail in relation to the rôle of the liver in fat metabolism and to the effect of choline in the prevention and cure of fatty livers.

(*d*) *Cholesterol.* The livers of animals in both series showed no change in the free cholesterol content. On the other hand, striking increases in the amount of cholesteryl esters in the livers began to appear within seven hours of feeding and the increases continued in linear fashion to the end of the experiment. This change therefore represents the first stage in the production of the cholesterol fatty liver by cholesterol feeding. Choline had no marked effect in preventing this increase.

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REFERENCES.

Artom (1933). *Arch. Fisiol.* **32**, 57.
Best (1934). *Lancet*, i, 1274.
—— Channon and Ridout (1934). *J. Physiol.* **81**, 409.
Channon, Irving and Smith (1934, 1). *Biochem. J.* **28**, 840.
—— —— —— (1934, 2). *Biochem. J.* **28**, 1807.
—— and Wilkinson (1934). *Biochem. J.* **28**, 2026.
Chanutin and Ludewig (1933). *J. Biol. Chem.* **102**, 57.
Hartley (1909). *J. Physiol.* **38**, 353.
Imrie (1914). *J. Path. Bact.* **19**, 245.
Leathes (1925). Cited from Leathes and Raper, *The fats*. (Longmans, Green and Co., London.)
—— and Meyer-Wedell (1909). *J. Physiol.* **38**, Proc. xxxviii.
Okey (1933, 1). *Proc. Soc. Exp. Biol. Med.* **30**, 1003.
—— (1933, 2). *J. Biol. Chem.* **100**, lxxv.
Sinclair (1929). *J. Biol. Chem.* **82**, 117.
Terroine (1919). *Ann. Sci. Nat. Zool.* **4**, 1.
—— and Bélin (1929). *Bull. Soc. Chim. Biol.* **9**, 12.

XXIII. LABILE GLUTAMINE PEPTIDES, AND THEIR BEARING ON THE ORIGIN OF THE AMMONIA SET FREE DURING THE ENZYMIC DIGESTION OF PROTEINS.

By JAMES MELVILLE.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

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That the ammonia (amide-N) given by proteins on acid hydrolysis comes in part from acid amides present in the molecule has been recently proved by the isolation after enzymic digestion of *l*-asparagine from edestin [Damodaran, 1932] and of *d*-glutamine from gliadin [Damodaran *et al.*, 1932]. In the latter research the amount of *d*-glutamine present at various stages was estimated by the indirect method of Chibnall and Westall [1932]. At the end of the digestion of the gliadin the amount present was equivalent to 43 % of the total amide-N. More than half of this was lost during precipitation of bases with phosphotungstic acid and the final yield of crystalline *d*-glutamine was equivalent to only about 5 % of the total amide-N. Damodaran *et al.* [1932] concluded that the low yield was due in large part to the unstable nature of *d*-glutamine in aqueous solution. They pointed out however that the indirect determination of glutamine amide-N might include labile peptides of *d*-glutamine as well as this amide. Since Chibnall and Westall [1932] had shown that *l*-leucyl-*d*-glutamine was stable under the hydrolytic conditions employed it was suggested that these labile peptides might be those resembling *d*-glutamine itself in having a free amino-group in the γ-position with respect to the amide group.

That relatively large amounts of labile glutamine peptides might indeed be present under certain conditions in the enzymic digests of proteins followed from the unpublished work of Dr Jaaback carried out in this laboratory during 1932–33. Caseinogen was digested with trypsin, the products extracted with butyl alcohol and the distribution of glutamine amide-N determined by the indirect method of Chibnall and Westall. The monoamino-acid fraction, which would include both asparagine and glutamine, contained only one third of the glutamine amide-N present in the original digest. The other two thirds remained in the aqueous phase and if the validity of the amide hypothesis be assumed they must have represented labile peptides of glutamine of predominantly acidic or basic character.

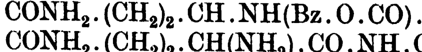
Synthesis of glutamine peptides of the second type mentioned above and a comprehensive study of their properties were therefore clearly desirable, but on account of the probably labile nature of the γ-amide group the problem was not easy of solution by the older methods of peptide synthesis. By a fortunate coincidence however Bergmann and Zervas [1932] published about this time a new method which offered some likelihood of success. In practice this has been found excellent for the purpose and a neutral dipeptide *d*-glutaminylglycine, an acidic dipeptide *d*-glutaminyl-*d*-glutamic acid and a neutral tripeptide *d*-glutaminylglycylglycine have been prepared. In all three the amide group is as labile as in *d*-glutamine itself and all exhibit to a lesser degree the abnormal reaction of this substance with nitrous acid during a determination of amino-N by the method of Van Slyke.

Since the method of Bergmann and Zervas [1932] permits the ready synthesis of *d*-glutamine from *d*-glutamic acid a sufficient amount of this amide has also been prepared to investigate for the first time a conclusion that can be drawn from the work of Damodaran *et al.* [1932] and of Jaaback (unpublished), that the ammonia which slowly accumulates during the enzymic digestion of proteins is due to the secondary decomposition of *d*-glutamine and *d*-glutaminyl peptides under the conditions employed and not to the presence of any specific amidase in the enzyme preparations. Opportunity has also been taken to investigate the hydrolysis of *d*-glutaminyl peptides by the yeast peptidases used in the digestion experiments, so that optimum conditions may be employed in future work.

Synthesis of glutamine peptides.

Treatment of *N*-benzylcarbonatoglutamic anhydride (I) with glycine ester on the lines of the synthesis of *d*-*iso*glutamine by Bergmann and Zervas [1932] gave analogously *N*-benzylcarbonato-*d*-α-glutamylglycine ethyl ester (II). Phosphorus pentachloride converted this into the γ-acid chloride (III) which reacted with ethereal ammonia to give *N*-benzylcarbonato-*d*-glutaminylglycine ethyl ester (IV). *N*-Benzylcarbonato-*d*-glutaminylglycine (V), obtained by hydrolysis of (IV), on hydrogenation in the presence of palladium yielded *d*-glutaminylglycine (VI). By substituting *d*-glutamic acid diethyl ester and glycylglycine ethyl ester for glycine ethyl ester in the above synthesis *d*-glutaminyl-*d*-glutamic acid[1] (VII) and *d*-glutaminylglycylglycine (VIII) were also prepared.

d-Glutamine was synthesised by a similar method. *N*-Benzylcarbonatoglutamic anhydride with sodium ethoxide gave *N*-benzylcarbonato-*d*-glutamic acid α-ethyl ester which was converted *via* the acid chloride to the γ-amide. Mild hydrolysis removed the ester group and the resulting *N*-benzylcarbonato-*d*-glutamine was catalytically reduced to *d*-glutamine. Experimental details of this synthesis are omitted, as one giving a better yield has since been described by Bergmann *et al.* [1933].

I. 

$$\underbrace{CO.(CH_2)_2.CH.NH(Bz.O.CO).CO.}_{O}$$

II. $CO_2H.(CH_2)_2.CH.NH(Bz.O.CO).CONH.CH_2.CO_2Et.$

III. $COCl.(CH_2)_2.CH.NH(Bz.O.CO).CO.NH.CH_2.CO_2Et.$

IV. $CONH_2.(CH_2)_2.CH.NH(Bz.O.CO).CO.NH.CH_2.CO_2Et.$

V. $CONH_2.(CH_2)_2.CH.NH(Bz.O.CO).CO.NH.CH_2.CO_2H.$

VI. $CONH_2.(CH_2)_2.CH(NH_2).CO.NH.CH_2.CO_2H.$

VII. $CONH_2.(CH_2)_2.CH(NH_2).CO.NH.CH(CO_2H).(CH_2)_2.CO_2H.$

VIII. $CONH_2.(CH_2)_2.CH(NH_2).CO.NH.CH_2.CO.NH.CH_2.CO_2H.$

N-Benzylcarbonato-d-*α-glutamylglycine ethyl ester.* 10 g. of *N*-benzylcarbonato-*d*-glutamic anhydride [Bergmann and Zervas, 1932] were dissolved in dry chloroform and 10 g. of glycine ethyl ester added. After 5 hours the solution was washed with dilute hydrochloric acid, dried with anhydrous sodium sulphate and the chloroform removed *in vacuo*. The residue was taken up in 10 ml. of absolute alcohol and left overnight in the ice-chest, when 6·5 g. of material crystallised out (47 % on the anhydride taken). Recrystallised from a small volume of alcohol it melted at 124°. (Found: N, 7·5 %. $C_{17}H_{22}O_7N_2$ requires N, 7·65 %. 0·174 g. required 4·75 ml. of 0·1 *N* NaOH; calculated, 4·75 ml.)

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[1] It is unfortunate that Bergmann and Zervas [1932] refer to glutamylglutamic acid as "Glutaminylglutaminsäure" instead of "Glutamylglutaminsäure" (*cf.* Chem. Zentr. 1923, III, 1084) as it will lead to confusion in German with the glutamine peptide glutaminylglutamic acid described in the present paper.

N-*Benzylcarbonato*-d-*glutaminylglycine ethyl ester.* 1·5 g. of the above ester in 30 ml. of dry chloroform were treated at $-5°$ with 1 g. of pulverised phosphorus pentachloride. After 5 mins. of vigorous shaking the chloroform solution was decanted and evaporated at the pump for 10 mins. to remove free hydrochloric acid. It was then added to a cold saturated ethereal solution of ammonia. After allowing to stand for about an hour the precipitate was filtered off and after removal of the solvent was thoroughly triturated with water. The insoluble residue crystallised well from alcohol. Yield 0·9 g.; M.P. 167°. (Found: N, 11·5; amide-N (Sachsse) 3·8 %. $C_{17}H_{23}O_6N_3$ requires N, 11·5; amide-N, 3·8 %.)

N-*Benzylcarbonato*-d-*glutaminylglycine.* 2·9 g. of the above half amide half ester were dissolved in 100 ml. of alcohol, aqueous sodium hydroxide was added to the first blue colour of thymolphthalein, the solution cooled to 20° and 8 ml. of *N* NaOH added. After standing for 90 mins. a slight excess of acid was added, the solution filtered and the filtrate evaporated to 40 ml. at the pump. This was kept at 0° overnight when 2·1 g. of crystalline *N*-benzylcarbonatoglutaminylglycine were obtained, corresponding to a 78 % yield. The material was not further purified. (Found: N, 12·4; amide-N, 4·0 %. $C_{15}H_{19}O_6N_3$ requires N, 12·5; amide-N, 4·1 %.)

d-*Glutaminylglycine.* 2 g. of the above compound dissolved in 70 ml. of aqueous methyl alcohol were hydrogenated in the presence of palladium. The evolution of CO_2 stopped after 20 mins., when the solution was filtered from the catalyst, brought to neutrality with ammonia and evaporated at a temperature not exceeding 40° to 10 ml. The dipeptide crystallised out on addition of alcohol and was purified by recrystallisation from aqueous alcohol. Yield 0·7 g. (Found: C, 41·3; H, 6·4; N, 20·7; amide-N, 6·9 %. $C_7H_{13}O_4N_3$ requires C, 41·4; H, 6·4; N, 20·7; amide-N, 6·9 %.) For a 4·8 % solution in water the observed rotation was $\alpha_D^{18°} + 3{\cdot}65°$, whence $[\alpha]_D^{18°} = +76°$.

d-*Glutaminyl*-d-*glutamic acid.* *N*-Benzylcarbonato-*d*-α-glutamyl-*d*-glutamic acid diethyl ester was prepared by the method of Bergmann and Zervas [1932], the yield being 65 % as against the quoted yield of 37 %. Following the procedure outlined above this was converted in 80 % yield into *N*-benzylcarbonato-*d*-glutaminyl-*d*-glutamic acid diethyl ester. Recrystallised from alcohol this melted at 181°. (Found: N, 9·1; amide-N, 3·0 %. $C_{22}H_{31}O_8N_3$ requires N, 9·0; amide-N, 3·0 %.) 2 g. of this amide ester were dissolved in 80 ml. of alcohol, a few drops of thymolphthalein solution added and then 4·6 ml. of *N* NaOH. The mixture was kept at 37° until an aliquot showed by back-titration with acid that the hydrolysis was complete (5–10 hours). 1·1 g. of *N*-benzylcarbonato-*d*-glutaminyl-*d*-glutamic acid were obtained. (Found: N, 10·1; amide-N, 3·3 %. $C_{18}H_{23}O_8N_3$ requires N, 10·3; amide-N, 3·4 %.) Reduction was carried out in the usual way. The dipeptide crystallised during the reduction and was separated from the catalyst by washing with a considerable volume of warm water. On evaporating the solution and washings *in vacuo* to a small volume, *d*-glutaminyl-*d*-glutamic acid crystallised out in 80 % yield. Purification was effected by dissolving in the smallest possible volume of water at 50°, evaporating *in vacuo* till the peptide began to crystallise and leaving overnight in the ice-chest. (Found: C, 43·6; H, 6·2; N, 15·5; amide-N, 5·1 %. $C_{10}H_{17}O_6N_3$ requires C, 43·7; H, 6·2; N, 15·5; amide-N, 5·2 %.) For a 4·8 % solution in *N* HCl the observed rotation was $\alpha_D^{18°} + 0{\cdot}63°$, whence $[\alpha]_D^{18°} = +15°$.

d-*Glutaminylglycylglycine*. Glycylglycine ethyl ester, prepared from glycine ethyl ester by the method of Fischer and Fourneau [1901] reacted with *N*-benzylcarbonato-*d*-glutamic anhydride to give *N*-benzylcarbonato-*d*-α-glutamylglycylglycine ethyl ester, M.P. 136°, in 54 % yield. (Found: N, 9·9 %. $C_{19}H_{25}O_8N_3$ requires N 9·9 %. 0·0198 g. required 3·15 ml. *N*/70 NaOH; calculated 3·30 ml.) The ester did not dissolve readily in chloroform but it was possible to prepare a supersaturated solution for the reaction with phosphorus pentachloride. Treatment with ammonia gave *N*-benzylcarbonato-*d*-glutaminylglycylglycine ethyl ester in 57 % yield. Recrystallised from alcohol this melted at 150–152°. (Found: N, 13·0; amide-N, 3·0 %. $C_{19}H_{26}O_7N_4$ requires N, 13·3; amide-N, 3·3 %). No crystalline product could be isolated after hydrolysis of the ester grouping, so methyl alcohol was added and the reduction carried out forthwith. The yield of the tripeptide was poor and there was insufficient material to effect complete purification. The best sample gave the following analysis. (Found: C, 40·8; H, 6·2; N, 19·3 %. $C_9H_{16}O_5N_4$ requires C, 41·5; H, 6·15; N, 21·5 %.)

d-iso*Glutamine*. A sample of this prepared by the method of Bergmann and Zervas [1932] gave correct analytical figures for N and amide-N but did not give satisfactory results when titrated electrometrically [*cf*. Melville and Richardson, 1935], and it was necessary to purify the material by recrystallisation three times from aqueous acetone before sufficiently good constants could be obtained. Since the original sample gave an abnormally high amino-N (Van Slyke) value and yielded a considerable amount of ammonia on hydrolysis at p_H 4 it was suspected that the product was considerably contaminated with *d*-glutamine, *i.e.* the anhydride ring had opened to give both the α- and γ-amides, recalling the unexpected results obtained by Bergmann *et al.* [1934], who found that the condensation of benzylcarbonato-*l*-aspartic anhydride and *l*-tyrosine methyl ester gave the β- and not the expected α-dipeptide. Since the point is of importance in any synthesis utilising either of these anhydrides as starting material, further information as to the composition of Bergmann and Zervas' *iso*glutamine was desirable. The anhydride was treated with ammonia according to their directions and the product (5·8 g.) catalytically reduced. This material was crystallised from aqueous alcohol, two crops being obtained, weighing 2·1 g. and 0·5 g. respectively, a total yield of 89 %. The first crop on analysis gave a glutamine amide-N value of 10 % and the second of 35 % of the total amide-N. It was found that the *iso*glutamine could be purified either by heating its aqueous solution at 100° for an hour or by recrystallisation from aqueous acetone. Thus 1·4 g. of the first crop mentioned above were dissolved in water and heated in a boiling water-bath for one hour. On the addition of alcohol 1·1 g. of material which gave only 2 % of its total amide-N as glutamine amide-N were recovered. (Found: N, 19·2 %. $C_5H_{10}O_3N_2$ requires 19·2 %.) For a 5·4 % solution in water the observed rotation was $\alpha_D^{18°}$ +1·05° whence $[\alpha]_D^{18°} = +19·1°$. Bergmann and Zervas give $[\alpha]_D^{20°} = +21·1°$, so that heating with water may have brought about slight racemisation.

One recrystallisation of another sample from aqueous acetone reduced the glutamine amide-N value from 12 % to 3 % of the total amide-N. From these results it is clear that a substance containing labile amide-N, undoubtedly *d*-glutamine, was present in the original *iso*glutamine preparations, and that when the anhydride ring of *N*-benzylcarbonato-*d*-glutamic anhydride was opened by ammonia both α- and γ-amides were formed in the proportion of about six to one.

It is reasonable to assume both from the figures given in the last column of Table I and from the data given in Table IV that in the case of the glutaminyl peptides described if any of the γ-peptide derivatives were formed during the opening of the anhydride ring they were successfully removed during the crystallisations at subsequent stages. But the possibility that the condensation between anhydride and amino-acid ester does not always go completely in one direction should not be overlooked in future work.

The properties of d-*glutamine and* d-*glutaminyl peptides.*

As was expected the three peptides whose synthesis has just been described showed in lesser degree the abnormalities which distinguish *d*-glutamine from all other amino-acids. The results of an investigation are given in Table I. *d*-Glutaminylglycine and *d*-glutaminyl-*d*-glutamic acid both yield about 90 % of their amide-N as ammonia on hydrolysis at p_H 4, and although the abnormal reaction with nitrous acid is not so marked as with *d*-glutamine itself the values obtained are 35–50 % higher than calculated.

Table I. *Properties of* d-*glutamine and related compounds.*

	Amino-N (% total N)		Ammonia-N given on hydrolysis at p_H 4 (% total amide-N)
	Van Slyke	Calculated	
d-Glutamine	95	50·0	86·0
d-*iso*Glutamine	55	50·0	2·0
l-Asparagine*	50	50·0	1·0
l-Leucyl-*d*-glutamine*	33	33·3	2·8
d-Glutaminylglycine	49	33·3	91·0
d-Glutaminyl-*d*-glutamic acid	45	33·3	87·0
d-Glutaminylglycylglycine	32	25·0	65·0

* Values from Chibnall and Westall [1932].

These results emphasise the inaccuracy of the Van Slyke amino-N values obtained during the course of an enzymic digestion of proteins such as gliadin which yield relatively large amounts of ammonia and glutamic acid on acid hydrolysis. Further, the abnormalities due to glutamine and glutaminyl peptides cannot, as was assumed by Damodaran *et al.* [1932], be corrected by a determination of glutamine amide-N, since from the data available there appears to be no correlation between this value and the amino-N (Van Slyke) value. The abnormal Van Slyke values of these compounds may however explain a point raised by Calvery [1933]. When comparing amino-N (Van Slyke) with an alcohol (NaOH) titration he finds that in a digest of egg-albumin there is equivalence to within 3 % of amino- and carboxyl groups liberated and assumes that proline peptide bonds are not split to any marked degree. Egg-albumin contains, in terms of total N, 8·5 % glutamic acid-N and 7·3 % ammonia-N so that it is reasonable to assume that considerable amounts of glutamine and glutaminyl peptides are present in the later stages of digestion, whose effect on the Van Slyke values would compensate for the hydrolysis of proline peptides.

The stability of d-*glutamine and glutamine peptides in aqueous solution at* 37°.

The stability of *d*-glutamine at p_H values of 1·8, 7·0, 7·8 and 8·3, the optimum reactions for pepsin, aminopolypeptidase, dipeptidase and trypsin respectively, has been studied both in the presence and absence of these enzymes. (Tables II and III.) In aqueous solution at p_H 1·8 and 37° the breakdown was quite rapid, over 80 % of the total amide-N being liberated as ammonia in two days. At p_H 7·8 and p_H 8·3 approximately 40 % breakdown had occurred in two days, while at p_H 7·0 this degree of splitting was attained after about 3 days. Similar experiments with *l*-asparagine and *l*-leucyl-*d*-glutamine showed that they were almost completely stable under these conditions. Insufficient material was available to determine the stability of the labile glutamine peptides at different p_H values, but an experiment at p_H 8·3 showed that *d*-glutaminyl-*d*-glutamic acid was split at approximately the same rate as *d*-glutamine itself. No appreciable glutaminase activity was present in commercial preparations of pepsin and trypsin, or in a week-old preparation of yeast peptidase. It will be seen that these results offer strong presumptive evidence for the theory that the ammonia formed during the digestion of proteins by the above-mentioned enzymes comes from the spontaneous breakdown of *d*-glutamine and labile glutamine peptides.

It is to be noted that Grassmann and Mayr [1932] found that a fresh yeast autolysate exhibited glutaminase activity. None was observed in the week-old preparation used in the present case, owing either to the fact that this enzyme is rapidly inactivated in ice-cold solution or to the different strains of yeast employed in English and German breweries.

Chibnall and Westall [1932] showed that when *d*-glutamine in aqueous solution was heated at 100° for 3 hours at p_H 4 not only was there a large liberation of ammonia from the amide group but also a fall in the amino-N (Van Slyke) from 190 to 10–20 % of the theoretical value, suggesting that the liberated glutamic acid had been rapidly transformed into pyrrolidonecarboxylic acid. Since breakdown of *d*-glutamine in aqueous solution also takes place at 37° it was necessary to find out what effect this might have on the titrimetric technique used to follow the enzymic hydrolysis of the glutaminyl peptides, for the production of glutamic acid would lead to an increase in the acetone (NaOH) titre whereas its transformation into pyrrolidonecarboxylic acid, with no free amino-group, would leave matters undisturbed.

Actually an experiment at p_H 7·0 in which aliquots were taken at the same time intervals for estimation of ammonia and for acetone titration showed that there is no appreciable increase in titre (Table III). This is confirmed by the results of Grassmann and Mayr [1932] who studied the action of asparaginase on *d*-glutamine at p_H 8·2 and found no significant difference in titre after 48 hours, during which time, as can be seen from Table II, nearly 40 % of the *d*-glutamine

Table II. *Stability of* d-*glutamine at* p_H *values of 1·85, 7·83 and 8·3.*

The *d*-glutamine used contained 18·7 % N. p_H values were checked at the hydrogen electrode. All solutions were made up to 25 ml. and 5 ml. aliquots taken for analysis. Temperature 37°. The ammonia liberated was estimated by means of the micro-apparatus of Parnas and Heller [1924] and is expressed in ml. of *N*/70 HCl.

At p_H 1·85 Solution A contained 0·0493 g. of *d*-glutamine and Solution B 0·0497 g. of *d*-glutamine +0·05 g. of pepsin. Buffer solution *M*/10 glycine—HCl.

At p_H 7·83 Solution C contained 0·0468 g. of *d*-glutamine in *M*/20 phosphate buffer.

At p_H 8·3 Solution D contained 0·0506 g. of *d*-glutamine and Solution E 0·0505 g. of *d*-glutamine +0·05 g. trypsin in *M*/20 barbitone (veronal) buffer.

At p_H 1·85			At p_H 7·83		At p_H 8·3		
	NH_3 liberated			NH_3 liberated		NH_3 liberated	
Time in hours	Solution A	Solution B	Time in hours	Solution C	Time in hours	Solution D	Solution E
5	0·98	0·95	20	0·99	18	0·80	0·79
25	3·27	3·23	46	1·93	42	1·74	1·71
49	4·14	4·14	68	2·27	66	2·41	2·42
100 % splitting	4·70	4 73	—	4·46	—	4·85	4·84

Table III. *Stability of* d-*glutamine at* p_H *7·05.*

Solution A contained 0·0518 g. of *d*-glutamine made up to 15 ml. with *M*/40 phosphate buffer. 2 ml. aliquots were used for titration in acetone with 0·022 *N* alcoholic NaOH and for the estimation of free ammonia. Solution B contained 0·0396 g. of *d*-glutamine, Solution C 0·0402 g. of *d*-glutamine + 2 ml. of a week-old neutral autolysate of yeast in *M*/20 phosphate buffer. 5 ml. aliquots were used for analysis. Ammonia liberated is expressed in ml. of *N*/70 HCl.

Solution A				NH$_3$ liberated	
Time in hours	Acetone titre	NH$_3$ liberated	Time in hours	Solution B	Solution C
0	5·20	0·02	24	0·52	0·47
23	5·15	0·53	90	1·62	1·58
71	5·15	1·38	142	2·23	2·19
100 % splitting	—	3·3	—	3·8	3·87

must have been decomposed. It appears therefore that during the breakdown the glutamic acid must have been rapidly transformed into pyrrolidonecarboxylic acid.

Action of yeast peptidases on glutamine peptides.

Insufficient glutaminyl peptides were available to work out accurately a full p_H-activity curve with the mixture of peptidases obtained by plasmolysis of yeast with ethyl acetate and autolysis at p_H 6·5 [Grassmann, 1927]. Comparative studies have been made at p_H 6·3, 7·0 and 7·8 on the peptides *d*-glutaminylglycine, *d*-glutaminyl-*d*-glutamic acid and *l*-leucyl-*d*-glutamine with *dl*-leucylglycine as control. About 0·125 millimol. of the peptide was dissolved in 0·025 *M* phosphate buffer (at p_H 7·8, 0·05 *M* *iso*glutamine buffer solution was used [*cf.* Melville and Richardson, 1935]) and the calculated volume of 0·2 *N* NaOH added to bring the peptides to the same p_H. The requisite amount of enzyme solution, about 0·4 ml. which had previously been brought to the buffer p_H, was added and the solution made up to 10 ml. with buffer. All p_H values were checked at the hydrogen electrode. The hydrolyses were carried out at 37° and were followed by titration in 90 % acetone [*cf.* Willstätter and Waldschmidt-Leitz, 1921], using 0·022 *N* alcoholic NaOH as titrating agent and thymolphthalein as indicator. An experiment with the crude tripeptide *d*-glutaminylglycylglycine at p_H 7·0 showed that it was split at least as rapidly as *dl*-leucylglycylglycine.

Table IV. *Action of yeast peptidases on peptides of glutamine.*

				Increase in titre				
Dipeptide	p_H	Wt. in g.-millimols	ml. of 0·2 *N* NaOH	30 mins.	75 mins.	135 mins.	195 mins.	100 % splitting
d-Glutaminyl-glycine	6·3	0·126	—	0·60	0·90	1·00	—	1·14
	7·05	0·144	0·24	1·0	1·1	1·15	—	1·31
	7·8	0·119	0·40	0·20	0·70	1·0	—	1·08
d-Glutaminyl-*d*-glutamic acid	6·3*	0·131	0·65	0·75	0·90	1·10	—	1·19
	7·05	0·131	0·80	0·25	0·65	0·95	—	1·19
	7·8	0·124	1·0	0·10	0·35	0·50	—	1·08
l-Leucyl-*d*-glutamine	6·3	0·138	—	0·30	0·80	1·15	—	1·25
	7·05	0·133	—	0·20	0·55	0·95	—	1·21
	7·8	0·141	0·18	0·25	0·70	1·10	—	1·28
dl-Leucylglycine	6·3	0·259	—	0·10	—	—	0·95	1·18
	7·05	0·256	—	0·15	0·40	—	1·10	1·16
	7·8	0·253	0·30	0·20	0·70	1·05	—	1·10

*** 1 ml. of enzyme was used instead of 0·4 as in the other cases.**

The results given in Table IV show that all the peptides are rapidly split and suggest that p_H 7·0 or a little lower is the best reaction for the scission of peptides of *d*-glutamine with yeast peptidase, particularly as at this p_H the breakdown of *d*-glutamine itself is considerably retarded.

SUMMARY.

The three peptides *d*-glutaminylglycine, *d*-glutaminyl-*d*-glutamic acid and *d*-glutaminylglycylglycine have been synthesised by the method of Bergmann and Zervas [1932]. They are readily hydrolysed by yeast peptidases.

As was expected, they exhibit the labile characters of *d*-glutamine itself, *i.e.* they give abnormally high amino-N (Van Slyke) values and are unstable in aqueous solution at 100°. A determination of glutamine amide-N on an enzymic digest of a protein by the method of Chibnall and Westall [1932] therefore may include glutaminyl peptides as well as glutamine itself.

The stability of *d*-glutamine and glutaminyl peptides in aqueous solution at 37° and various p_H values has been determined. All the amide-N is hydrolysed to ammonia in 4–9 days, and the glutamic acid set free is transformed into pyrrolidonecarboxylic acid. The bearing that these observations have on the origin of the ammonia liberated during the enzymic digestion of proteins and also on the titrimetric technique generally used to follow the course of the digestion is briefly discussed.

I am indebted to Prof. A. C. Chibnall for suggesting this work and for constant advice and criticism. My thanks are also due to Dr J. M. Gulland for samples of *dl*-leucylglycine and *dl*-leucylglycylglycine and to the University of New Zealand for a Travelling Scholarship.

REFERENCES.

Bergmann and Zervas (1932). *Ber. deutsch. chem. Ges.* **65**, 1192.
— — and Salzmann (1933). *Ber. deutsch. chem. Ges.* **66**, 1288.
— — — and Schleich (1934). *Z. physiol. Chem.* **224**, 17.
Calvery (1933). *J. Biol. Chem.* **102**, 73.
Chibnall and Westall (1932). *Biochem. J.* **26**, 122.
Damodaran (1932). *Biochem. J.* **26**, 235.
— Jaaback and Chibnall (1932). *Biochem. J.* **26**, 1704.
Fischer and Fourneau (1901). *Ber. deutsch. chem. Ges.* **34**, 2868.
Grassmann (1927). *Z. physiol. Chem.* **167**, 202.
— and Mayr (1932). *Z. physiol. Chem.* **214**, 185.
Melville and Richardson (1935). *Biochem. J.* **29**, 187.
Parnas and Heller (1924). *Biochem. Z.* **152**, 1.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 2988.

XXIV. THE TITRATION CONSTANTS OF SOME AMIDES AND DIPEPTIDES IN RELATION TO ALCOHOL AND FORMALDEHYDE TITRATIONS OF AMINO-N.

BY JAMES MELVILLE AND GEORGE MAXWELL RICHARDSON.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

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MEASUREMENT of the titration constants of simple peptides is of biochemical interest for two reasons. Firstly, it provides information necessary for careful titrimetric estimation of amino-N in biological fluids or enzymic proteolysates [Richardson, 1934, 1, 2]. Secondly, it facilitates interpretation of protein titration curves by helping to define the change of titration constant which results from peptide formation [Cohn, 1931].

For gelatin hydrolysates and various synthetic dipeptides, suitable data are already available [Cannan and Muntwyler, 1930; Greenstein, 1933; and others]. There is therefore no difficulty in deducing the extent of error due to these substances in alkalimetric formaldehyde or alcohol titrations of amino-N [Richardson, 1934, 2]. Yet there exists an important group of substances containing amino-N about which there has been insufficient information hitherto. These, the amino-acid amides, may be quite plentiful in certain plant extracts and probably of enzymic proteolysates [*cf.* Melville, 1935]. Titration constants for certain typical derivatives have therefore been examined, together with data for some peptides of possibly distinctive behaviour. In addition, the usefulness of *iso*-glutamine as a readily synthesised buffer of value in peptidase experiments is described.

EXPERIMENTAL.

The glutamic acid derivatives were synthesised from *d*-glutamic acid by Bergmann's benzylchlorocarbonate method [Melville, 1935], while for other materials we are deeply indebted to Prof. Max Bergmann [Bergmann *et al.*, 1929; 1932]. In our experience, all materials required several recrystallisations to free them from traces of titratable contaminants, primarily affecting the alkaline curves. The glutamic acid derivatives, which showed this effect initially, yielded admirable data on recrystallisation [Melville, 1935, p. 182]. Other materials were not further recrystallised, and one of these, *d*-tyrosyl-*d*-arginine, has required suitable allowance for a titrated impurity (5 % of the group titre). Similar contaminants in *l*-*iso*asparagine and *d*-alanyl-*l*-proline had a sufficiently alkaline range (about 9·6, suggestive of free amino-acids) to give no serious interference in the constants.

Titration from a micro-burette was carried out with hydrogenated 0·1 *M* NaOH or HCl with the recognised precautions, the reagents being standardised against Na_2CO_3, potassium hydrogen phthalate and constant-boiling HCl as ultimate standards. Separate solutions were used for acid and alkaline curves except in the cases of *d*-glutaminyl-*d*-glutamic acid and *d*-tyrosyl-*d*-arginine. Where a preparation was not originally isoelectric, a suitable correction (not exceeding 2 %) has been applied to both curves. Attempts to titrate the benzylcarbonato intermediaries at a hydrogen electrode were naturally abortive, owing to catalytic reduction with characteristic decay of electrode potential.

The apparatus has been described elsewhere [Melville and Richardson, 1934]; for titration at considerable acidity, however, a constricted vessel of 2 ml. minimum capacity was used. Junction with saturated KCl solution was made smoothly *in situ* about 10 min. before titration, and wandering potentials in acid solution were minimised by careful selection of bridges. Occasional renewal of the KCl surface was effected, in prolonged titration at acid extremes, by discharging small measured volumes of KCl from the bridge, the small salt increments being included in the estimated ionic strength. Electrodes were freshly palladised, gold-plated platinum wires, and working temperature was $25 \pm 0{\cdot}02^\circ$. The reference potential assumed for a hydrogen electrode in 0·05 *M* potassium hydrogen phthalate was 0·2347 v. at this temperature [Clark, 1928]. Duplicate electrodes seldom disagreed by more than 0·0001 v.

As usual, observed titres were corrected for free acid and alkali at the p_H extremes, the simple Debye-Hückel relation modified by Simms [1926] being used to calculate hydrion concentrations from p_H; thus, $p_H = \log \frac{1}{[H^+]} - \log \gamma$, where $-\log \gamma = 0{\cdot}3\sqrt{\mu}$ (Simms quotes $0{\cdot}2\sqrt{2\mu}$). μ, the ionic strength, was estimated without inclusion of the zwitterion. Hydroxyl ion concentrations were obtained correspondingly, the ionic (activity) product of water being accepted as $10^{-13{\cdot}995}$ at 25° [Clark, 1928]. The calculated constants, G′ *etc.*, are titration constants [von Muralt, 1930] uncorrected for activity of the ions concerned. Thus since ionic strength was not constant during titration, a titre which provides the best carboxyl constant may differ slightly from that providing the best amino constant. Carboxyl and amino constants are both expressed as hydrion dissociation constants (Brønsted-Lowry) using the Sørensen logarithmic nomenclature, $p_{G'}$ *etc.*

Results.

In Table I, full data for *d*-glutamine and *d*-*iso*glutamine are quoted, data for other materials being suitably abbreviated. The possibility that the amides would suffer hydrolysis during titration has fortunately not been realised. Thus *d*-glutamine and its peptides are sufficiently stable even at 37° [Melville, 1935] to permit a reasonable titration; at 25°, steady titration produced the consistent data of Table I. Measurements on glycyl-*l*-proline and *d*-tyrosyl-*d*-arginine had been completed before Greenstein's paper [1933] appeared. Glycyl-*l*-proline gave good constants in accord with Greenstein's on an identical sample, so that further detail is not required (Table II). The samples of *d*-tyrosyl-*d*-arginine were apparently not identical, however, and results are more fully detailed as there is considerable divergence. The source of disparity is difficult to ascertain in the absence of more particulars than are given in his paper, but our constants seem consistent enough to be accepted (except that the fourth constant (guanidine) is impaired by the large free alkali correction and by a necessary assumption of 5 % of impurity of $p_{G'}$ 10·1).

In Table II, the mean constants of Table I are collected and compared with accepted constants for related compounds (expressed on a zwitterionic basis).

Table I. *Calculation of titration constants.*

Titrated solutions were all prepared by weight.

(*a*) *d*-Glutamine (mol. wt. 146) —COOH 3 ml. 0·25 *M* titrated with 0·197 *M* HCl.
—NH_2 10 ml. 0·037 *M* „ 0·103 *M* NaOH.

(*b*) *d*-*iso*Glutamine (146) —COOH 10 ml. 0·030 *M* titrated with 0·103 *M* HCl.
—NH_2 10 ml. 0·032 *M* „ 0·103 *M* NaOH.

(*c*) *l*-*iso*Asparagine monohydrate (150) —COOH 10 ml. 0·056 *M* titrated with 0·0995 *M* HCl.
—NH_2 10 ml. 0·049 *M* „ 0·101 *M* NaOH.

(*d*) *d*-Alanyl-*l*-proline (186) —COOH 10 ml. 0·034 *M* titrated with 0·0995 *M* HCl.
—NH_2 10 ml. 0·035 *M* „ 0·101 *M* NaOH.

(*e*) *d*-Glutaminylglycine (203) —COOH 2 ml. 0·21 *M* titrated with 0·103 *M* HCl.
—NH_2 10 ml. 0·042 *M* „ 0·103 *M* NaOH.

(*f*) *d*-Glutaminyl-*d*-glutamic acid (275). 3·6 ml. 0·10 *M* solution in 0·2 *M* HCl titrated with 0·103 *M* NaOH (titres corrected for excess HCl).

(*g*) *d*-Tyrosyl-*d*-arginine (337). —COOH 10 ml. 0·021 *M* titrated with 0·0995 *M* HCl.
Other groups. Back-titration with 0·101 *M* NaOH (corrected for excess HCl).

	—COOH						—NH_2					
				Corrections						Corrections		
	p_H	Titre ml.	μ	Acidity ml.	Over-lap ml.	$p_{G'}$	p_H	Titre ml.	μ	Alkal. ml.	Over-lap ml.	$p_{G'}$
(*a*)	3·301	0·26	0·018	−0·01	—	2·161	8·048	0·27	—	—	—	9·134
	3·035	0·47	0·029	0·02	—	2·176	8·393	0·55	—	—	—	9·130
	2·854	0·67	0·038	0·03	—	2·174	8·630	0·85	—	—	—	9·133
	2·708	0·89	0·047	0·05	—	2·176	8·804	1·14	—	—	—	9·131
	2·547	1·17	0·058	0·07	—	2·174	8·977	1·46	—	—	—	9·135
	2·387	1·52	0·068	0·11	—	2·177	9·126	1·76	—	—	—	9·136
	2·223	1·93	0·079	0·18	—	2·176	9·301	2·12	—	—	—	9·133
	2·044	2·42	0·090	0·31	—	2·167	9·498	2·48	—	—	—	9·137
	1·888	2·88	0·099	0·48	—	2·154	10·002	3·15	0·024	0·01	—	9·128
		(3·70)	end-point					(3·56)				
(*b*)	4·865	0·23	—	—	—	3·803	7·270	0·61	—	—	—	7·886
	4·610	0·39	—	—	—	3·805	7·490	0·89	—	—	—	7·891
	4·344	0·65	—	—	—	3·808	7·649	1·15	—	—	—	7·884
	4·112	0·96	0·008	−0·01	—	3·804	7·811	1·43	—	—	—	7·886
	3·921	1·26	0·012	0·01	—	3·806	7·989	1·76	—	—	—	7·880
	3·751	1·55	0·013	0·02	—	3·806	8·170	2·07	—	—	—	7·880
	3·564	1·86	0·015	0·03	—	3·805	8·547	2·57	—	—	—	7·886
	3·388	2·14	0·017	0·06	—	3·803	8·782	2·78	—	—	—	7·882
	3·151	2·45	0·019	0·10	—	3·799	—	(3·13)	—	—	—	—
		(2·88)	end-point									
(*c*)	4·295	0·22	—	—	—	2·976	6·716	0·21	—	—	—	8·026
	3·688	0·80	0·012	−0·02	—	2·976	6·998	0·39	—	—	—	8·022
	3·473	1·20	0·017	0·04	—	2·976	7·551	1·14	—	—	—	8·021
	3·256	1·71	0·023	0·07	—	2·971	7·840	1·79	—	—	—	8·020
	3·051	2·29	0·028	0·12	—	2·968	8·043	2·31	—	—	—	8·020
	2·832	3·00	0·034	0·22	—	2·971	8·289	2·92	—	—	—	8·022
	2·590	3·81	0·038	0·41	—	2·975	8·622	3·60	—	—	—	8·020
		(4·80)	end-point					(4·50)				
(*d*)	4·105	0·25	0·006	−0·01	—	3·04	7·621	0·46	—	—	—	8·38
	3·706	0·55	0·013	0·02	—	3·03	7·841	0·70	—	—	—	8·38
	3·459	0·88	0·024	0·04	—	3·04	8·039	0·98	—	—	—	8·38
	3·002	1·69	0·045	0·13	—	3·03	8·345	1·50	—	—	—	8·38
	2·766	2·22	0·051	0·25	—	3·04	8·547	1·87	—	—	—	8·38
	2·475	2·88	0·057	0·51	—	3·03	8·910	2·44	—	—	—	8·37
		(3·03)	end-point					(3·14)				

Table I (*cont.*).

	—COOH						—NH_2					
				Corrections						Corrections		
	p_{H}	Titre ml.	μ	Acidity ml.	Over-lap ml.	$p_{G'}$	p_{H}	Titre ml.	μ	Alkal. ml.	Over-lap ml.	$p_{G'}$
(*e*)	4·196	0·33	—	—	—	3·14	6·702	0·53	—	—	—	7·52
	3·742	0·82	—	—	—	3·15	7·131	1·17	—	—	—	7·52
	3·243	1·83	0·047	-0·03	—	3·14	7·500	1·97	—	—	—	7·52
	2·897	2·67	0·057	0·07	—	3·15	7·700	2·43	—	—	—	7·52
	2·700	3·11	0·061	0·12	—	3·15	8·124	3·23	—	—	—	7·52
	2·280	3·93	0·066	0·35	—	3·15	8·713	3·79	—	—	—	7·51
		(4·06) end-point						(4·03)				
(*f*)	2·488	0·35	0·092	+0·30	-0·04	3·15	6·871	7·29	—	—	—	7·63
	2·791	0·97	0·086	0·16	0·08	3·14	7·135	7·61	—	—	—	7·63
	3·126	1·77	0·078	0·08	0·18	3·14	7·507	8·25	—	—	—	7·62
	3·301	2·22	0·075	0·05	0·26	3·14	7·710	8·64	—	—	—	7·62
	3·480	2·67	0·071	0·04	0·38	3·14	8·099	9·30	—	—	—	7·63
	3·794	3·45	0·066	0·02	0·70	3·15	8·625	9·85	—	—	—	7·62
		(3·395) end-point						(10·15)				
	3·958	3·85	0·064	+0·02	+0·45	4·38	—	—	—	—	—	—
	4·154	4·33	0·061	0·01	0·30	4·39	—	—	—	—	—	—
	4·325	4·77	0·059	0·01	0·21	4·38	—	—	—	—	—	—
	4·533	5·25	—	—	0·13	4·38	—	—	—	—	—	—
	4·923	5·99	—	—	0·05	4·38	—	—	—	—	—	—
	5·459	6·53	—	—	—	4·38	—	—	—	—	—	—
		(6·79)										
	—COOH						—OH					
(*g*)	—	(2·05)	—	—	—	—						
	3·461	2·37	0·030	-0·05	—	2·64	9·192	2·38	—	—	+0·02	9·79
	3·163	2·61	0·031	0·10	—	2·63	9·412	2·60	—	—	0·0	9·78
	2·965	2·86	0·032	0·16	—	2·63	9·656	2·88	0·040	-0·01	0·04	9·80
	2·710	3·27	0·036	0·29	—	2·63	9·861	3·18	0·045	0·02	0·07	9·78
	2·451	3·84	0·040	0·56	—	2·63	10·093	3·46	0·046	0·03	0·12	9·81
		(4·10) end-point						(4·00)				
	—NH_2						Guanidine					
	6·835	0·33	—	—	—	7·54	11·03	4·39	0·050	0·27	0·0	12·23
	7·111	0·54	—	—	-0·01	7·55	11·23	4·63	0·051	0·43	-0·04	12·29
	7·374	0·81	—	—	0·01	7·55	11·44	5·02	0·053	0·71	0·06	12·29
	7·650	1·12	—	—	0·02	7·56	11·68	5·77	0·055	1·27	0·08	12·26
	8·298	1·76	—	—	0·07	7·56	—	(6·00)	—	—	—	—
		(2·00) end-point										

Discussion.

Effect of structure on $p_{K'}$[1]. Many factors, not solely structural, take part in promoting hydrion dissociation. It is, however, a commonplace that kationoid groups in a molecule (—C_6H_5, —I, —COOH, —$COOC_2H_5$, —$CONH_2$, —NH_4^+) will favour hydrion dissociation (displace $p_{K'}$ to more acid regions), while anionoid groups (—CH_3, —COO^-, —NH_2) will retard it, the effect varying with the number, the potency and especially the proximity of the groups [see Allan *et al.*, 1926; Cohn, 1931]. Upon this basis, ample explanation of the major displacements of $p_{K'}$ in Table II is provided.

Thus, *glutamine* differs structurally from α-amino-*n*-butyric acid by a kationoid group, —$CONH_2$, in the γ-position, this distant group effecting a small decrease of both $p_{K'}$ (Table II); an enhanced effect with asparagine illustrates

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[1] For purposes of discussion, $p_{G'}$ is accepted as equivalent to $p_{K'}$. The approximation is small compared with the magnitudes discussed and the unspecified effects of salt concentration. Existence of optical isomerism is ignored as this is presumably without influence on $p_{K'}$.

Table II. *Titration constants at 25°.*

	—COOH	—COOH	$—NH_2$	—OH	Guan.	Reference
α-Amino-*n*-butyric acid	2·55	—	9·60	—	—	Czarnetzky and Schmidt [1932]
Glutamine (γ-amide)	2·17	—	9·13	—	—	This paper
Asparagine (β-amide)	2·02	—	8·80	—	—	Chibnall and Cannan [1930]
β-Hydroxyasparagine	2·12	—	8·26	—	—	Chibnall and Cannan [1930]
Glutamic acid	2·19	4·25	9·66	—	—	Kirk and Schmidt [1929]
*iso*Glutamine (α-amide)	3·81	—	7·88	—	—	This paper
*iso*Asparagine (α-amide)	2·97	—	8·02	—	—	This paper
α-Hydroxyasparagine	2·31	—	7·17	—	—	Chibnall and Cannan [1930]
Glutaminylglycine	3·15	—	7·52	—	—	This paper
Glutaminylglutamic acid	3·14	4·38	7·62	—	—	This paper
Glycylproline	2·84	—	8·53	—	—	This paper
Alanylproline	3·04	—	8·38	—	—	This paper
Alanylalanine	3·17	—	8·42	—	—	Simms [1928]
Phenylalanylarginine	2·66	—	7·57	—	12·4	Greenstein [1933]
Tyrosylarginine	2·63	—	7·55	9·80	12·3	This paper
,,	2·65	—	7·39	9·36	11·6	Greenstein [1933]

the result of structural proximity. The comparable behaviour of a kationoid —COOH group is shown in the first carboxyl constant of *glutamic acid*. The second carboxyl constant, however, is under the opposing influences of the anion, $—COO^-$, and a kation $—NH_3^+$, and is not greatly removed from the constants of fatty acids ($p_{K'}$ 4·7), while the amino constant of glutamic acid is increased over that of glutamine owing to the replacement of the kationoid $—CONH_2$ group by the anion $—COO^-$. In the case of iso*glutamine*, the carboxyl group is rather distant from the kationoid $—CONH_2$ group and the kation $—NH_3^+$ and is consequently not so much stronger than a fatty acid; but in *iso*asparagine there is considerable enhancement. The amino-group of *iso*glutamine is in the α-position to a kationoid $—CONH_2$ group (as compared with the α-$—COO^-$ anion of glutamine) and far removed from a $—COO^-$ anion; hence its $p_{K'}$ is displaced considerably in an acid direction. In *iso*asparagine, a corresponding displacement is retarded by the greater proximity of the $—COO^-$ anion. In the *hydroxyasparagines* similar considerations, including an effect of kationoid hydroxy-groups, are involved [Chibnall and Cannan, 1930].

There is no need for a detailed extension of the argument, but a few points require mention:

(*a*) A *dipeptide* may be regarded as a substituted amide R.CONH (CHR'. COO^-), the substituent carrying an α-$—COO^-$ anion (or kationoid —COOH) with perhaps further complicating groups. As a simple case, α-aspartylaspartic acid, $p_{K'_{NH_2}}$ 8·26 (from aspartylaspartic anhydride [Greenstein 1931]) is a substituted α-asparagine (8·02); whereas aspartylglycine, $p_{K'_{NH_2}}$ 9·07 [Simms, 1928][1], and β-aspartyltyrosine, $p_{K'_{NH_2}}$ 8·92 [Greenstein, 1933; Bergmann *et al.*, 1934] are substituted β-asparagines (8·80). Compared with the amides, each peptide shows a slight increase of $p_{K'_{NH_2}}$ due to substituent $—COO^-$ anions. At the same time, however, it is the position of the —CONH— group which is most powerful in determining the magnitude of the peptide $p_{K'_{NH_2}}$.

(*b*) Therefore, a peptide carrying a $—CONH_2$ group in addition to its typical —CONHR group must be expected to show an abnormally low $p_{K'_{NH_2}}$. Indeed the displacement of $p_{K'_{NH_2}}$ in the glutaminyl peptides is not exceeded in any amino-N compound of important biological distribution and has been equalled only when both residues of a dipeptide have contained kationoid groups (phenylalanylarginine, 7·57, tyrosyltyrosine 7·68 *etc.*, also cystine 7·5, 9·0). The possibilities of further depression by coupling such residues to glutamine, or particularly by preparing derivatives of α-hydroxyasparagine ($p_{K'_{NH_2}}$ 7·17) or β-hydroxyglutamine, should not be overlooked. In the meantime, it is likely that the mean $p_{K'_{NH_2}}$ for pepsin proteolysates at 7·9 [Cannan and Muntwyler, 1930] will be lower for proteolysates from other sources than gelatin which contains a minimum of kationoid groups. Of course, complexity of the polypeptides also operates to decrease the $p_{K'}$ (as in glycine hexapeptide, $p_{K'}$ 7·6 [Stiasny and Scotti, 1930]).

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[1] The exact structure of this peptide was not described, though Fischer's indeterminate synthesis from fumaryldiglycine was presumably employed. A paper from the same laboratory describes the preparation as stable to erepsin [Levene *et al.*, 1926], affording presumptive evidence of β-linkage [Bergmann *et al.*, 1934].

(*c*) It is noteworthy that introduction of a *p*-hydroxy-group into phenylalanine produces a negligible fall of $p_{K'_{NH_2}}$ in the resulting tyrosine (9·23 becomes 9·15). As it is unlikely that a different situation exists in their peptides (particularly after ionisation of the hydroxyl group), the analogy between our constants for tyrosylarginine and those for phenylalanylarginine (Table II) is satisfying. Again the hydroxyl constant of tyrosine (10·2) is not greatly decreased in the peptide, and resembles that of the similarly placed group in tyrosyltyrosine (9·80 [Greenstein, 1932]). Thus our new data show a uniformity previously lacking.

(*d*) There seems no essential difference between the acylproline and ordinary peptide linkages in their effect on $p_{K'}$.

Influence on the estimation of biological amino-N. In a detailed discussion of alcohol and formaldehyde titrations of biological fluids, it was shown [Richardson, 1934, 2] that a satisfactory and convenient indicator-point in the case of amino-acids (mean $p_{K'}$ 9·6) was the first visible colour of phenolphthalein (p_H 8·4). Now this discussion assumed a working temperature of 25°; at 15° the mean $p_{K'_{NH_2}}$ is more truly given at 9·9 with no accompanying change in the indicator range, so that the end-point could even be a little more alkaline than p_H 8·4. The more acid end-point favoured by Brown [1923] for his formaldehyde titration by a comparator method (phenol red, p_H 8·0) was due to the undue proportion of peptides or of amino-acids with kationoid groups which he investigated (*e.g.* asparagine, see below).

Now, the discussion quoted was concerned primarily with the single-indicator titration of simple amino-acids. It follows from the argument therein developed that where glutamine and asparagine may be present as bulk constituents, as in certain plant extracts, a lowered indicator-point is desirable to accommodate these lower values of $p_{K'}$ (9·13 and 8·80). Thus p_H 7·6 (phenol red) would be a likely point (Brown places it even lower for asparagine); yet at the same time the original indicator-points would not exclude any considerable portion of these amides from titration. A more important defect of the methods is that peptides (mean $p_{K'}$ 8·3) would largely escape estimation, as was noted in the discussion. A particularly serious example is presented by the peptides of glutamine and asparagine, or other peptides of abnormally low $p_{K'}$ (see previous section); in such cases the amino-groups would be 75 to 90 % titrated in the preliminary p_H adjustment and only the remaining 10 to 25 % would be included in the titration proper. The use of a lower indicator-point (p_H 6·5) to include peptide-N would automatically exclude the simpler amino-N. Conversely, a two-indicator method would include the desired groups but also various other unspecified substances.

Indeed, there is no titration method which can be safely applied without some foreknowledge of the chemical composition of the extract to be tested. Where such foreknowledge is lacking, a two-indicator titration of lead-treated extracts by the Lüers formaldehyde method merits further development [Richardson, 1934, 2], or, as a temporary expedient, parallel titration of aliquots to different indicator end-points may be used to find the optimum conditions (as with peptones [Brown, 1923]). The other common method for amino-N (Van Slyke) is unfortunately liable to other error [Richardson, 1934; Melville, 1935] and its use requires similar foreknowledge of the composition of the extract.

Influence on the study of peptic scission. An unusually low $p_{K'_{\mathrm{NH}_2}}$ in the dipeptides which result from protein scission will not adversely affect the customary Willstätter titrimetric estimation of the extent of hydrolysis [Richardson, 1934, 1]. On the other hand, the use of dipeptides with a low $p_{K'_{\mathrm{NH}_2}}$ as substrates in dipeptidase studies raises a novel problem as to effective buffering capacity. Thus for a scission experiment at the customary p_{H} of 7·4 to 7·8, it is clear that such a dipeptide ($p_{K'}$ 7·6) must first be brought to the appropriate p_{H} by addition of 0·4 to 0·6 equivalent of NaOH. When this is followed by scission of the peptide, this one group ($p_{K'}$ 7·6) will be progressively replaced by two groups of $p_{K'}$ 9 to 9·6, these groups sharing the added NaOH. This leads, in the absence of buffering, directly to an increase of p_{H} to 8·5 to 9. Conversely, with buffers present, these must be sufficiently effective to engage without great p_{H} change almost the whole of the NaOH previously held by the peptide.

In such cases it is necessary to modify the current procedure of Grassmann [1927] to allow greater constancy of p_{H} during hydrolysis. Indeed, much of the present evidence of p_{H} constancy seems to depend on the use of dipeptides ($p_{K'}$ 8·3) requiring little p_{H} adjustment, the buffering capacity of the enzyme preparation itself being partly responsible for the preservation of p_{H}. Certainly, Grassmann's early use [1927] of only 0·8 equivalent of NH_3– NH_4Cl buffer ($p_{K'}$ 9·3) at a p_{H} outside its effective buffering range (p_{H} 7·8) was grossly inadequate. At the same time, the subsequent general practice of using 0·03 *M* phosphate at p_{H} 7·4 to 7·8 to buffer peptides in 0·05 *M* to 0·1 *M* concentration [Bergmann *et al.*, 1934] is scarcely a sufficient improvement for critical p_{H} studies. Adequate buffering would need at least three equivalents of a buffer, whose $p_{K'}$ should be just alkaline to the experimental p_{H} in order to offer the maximum obstruction to the alkaline drift.

It is an added advantage if such a buffer is soluble in 90 % alcohol (or acetone), and is composed of some base or basic group and its salt (briefly because, in titration, the buffering range of a base is displaced by alcohol addition with respect to the phthalein indicator, so that encroachment of the buffer on the titration end-point is avoided [*cf.* Richardson, 1934, 1]; moreover, the temperature effects on the $p_{K'}$ of a basic buffer and of the titrated groups are comparable). In *iso*glutamine ($p_{K'}$ 7·9) these desirable properties are all combined and join with the convenient synthesis and stability of the material to mark it out for further study.

iso*Glutamine as a buffer for study of peptide scission.* Since the above criteria for buffering dipeptidase experiments are so admirably combined in *iso*glutamine, a few more details may be presented. *iso*Glutamine (α-glutamine) may be easily prepared from glutamic acid (for purity see Melville [1935, p. 182]) and has ample, though not great aqueous solubility. Used as a 0·03 *M* buffer for 0·01 *M* dipeptides, it does not interfere with alkalimetric acetone titration of amino-N, either by precipitating from solution or by encroaching on the end-point. In contrast the usual phosphate buffer is an acid substance with a $p_{K'}$ too low for adequate buffering against alkaline drifts and with insufficient solubility at the end-point. Barbitone, as an alternative, has a suitable $p_{K'}$ (8·2) and acetone solubility, but insufficient aqueous solubility (0·023 *M*) and being an acid is liable to interfere at the end-point.

Table III. *Stability of* d-iso*glutamine at 37°.*

0·124 g. *iso*glutamine adjusted to given p_H and made to 25 ml. (0·034 *M*) with phosphate or barbitone buffer. At intervals, 2 ml. were removed for NH_3 determination [*cf.* Melville, 1935, Tables II and III].

	NH₃ liberated (as ml. *M*/70 HCl)		
Time in hours	p_H 6·8	p_H 7·8	p_H 8·8
24	0·04	0·11	0·18
48	0·11	0·18	0·34
72	—	—	0·48
96	0·14	0·35	—
100 % scission	4·75	4·75	4·75

These features of *iso*glutamine would, of course, be valueless in presence of a latent tendency to hydrolysis of the α-amide group. Fortunately, Table III shows that this tendency is negligible under the conditions of ordinary experiment (it was interesting that simultaneous amino-N titrations showed no important increase in amino-N + NH_3 during liberation of NH_3, virtual evidence of ring closure). Moreover, as *d-iso*glutamine showed no hydrolysis whatever during two hours with mixed yeast peptidases under conditions giving 60 % scission of *d*-glutaminylglycine, no fear of hydrolytic scission of the buffer need normally be entertained in experiments of the usual duration.

Finally, data for the preparation of 0·03 *M* *iso*glutamine buffers are interpolated from the data of Table I and presented in Table IV. Similar data for *iso*asparagine ($p_{K'}$ 8·0) may be readily prepared from Table I, should *iso*glutamine prove unsuitable in unusual circumstances.

Table IV. *Preparation of* iso*glutamine buffers* (*for immediate use*).

M/30 buffer prepared from 0·487 g. pure *iso*glutamine with given volume of 0·1 *M* NaOH, made to 100 ml. p_H at 25°.

p_H	7·0	7·2	7·4	7·6	7·8	8·0	8·2	8·4	8·6	8·8
ml. 0·1 *M* NaOH	3·85	5·7	8·2	11·4	15·1	18·9	22·5	25·5	28·0	29·7

SUMMARY.

Titration constants at 25° have been determined for *d*-glutamine, *d-iso*-glutamine, *l-iso*asparagine, *d*-glutaminylglycine, *d*-glutaminyl-*d*-glutamic acid and *d*-alanyl-*l*-proline, and re-determined for *d*-tyrosyl-*d*-arginine and glycyl-*l*-proline. Factors influencing the magnitude of the respective $p_{K'}$ values are discussed. α-Amides and α-peptides possess constants characteristically different from their β- or γ-analogues.

In certain peptides the constants attributable to the amino-group are unusually low, and the influence of this fact on the titrimetric estimation of biological amino-N and on the study of peptidase action is discussed.

Advantages attending the use of *iso*glutamine as a buffer in peptidase studies are outlined, and the necessary data as to the composition and stability of the buffer mixtures are presented.

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REFERENCES.

Allan, Oxford, Robinson and Smith (1926). *J. Chem. Soc.* 405.
Bergmann and Zervas (1932). *Ber. deutsch. chem. Ges.* **65**, 1192.
—— —— Schleich and Leinert (1932). *Z. physiol. Chem.* **212**, 72.
—— —— Salzmann and Schleich (1934). *Z. physiol. Chem.* **224**, 17.
—— —— and du Vigneaud (1929). *Ber. deutsch. chem. Ges.* **62**, 1905.
Brown (1923). *J. Bact.* **8**, 245.
Cannan and Muntwyler (1930). *Biochem. J.* **24**, 1012.
Chibnall and Cannan (1930). *Biochem. J.* **24**, 945.
Clark (1928). The determination of hydrogen ions. (London.)
Cohn (1931). *Ergebn. Physiol.* **33**, 781.
Czarnetzky and Schmidt (1932). *Z. physiol. Chem.* **204**, 129.
Grassmann (1927). *Z. physiol. Chem.* **167**, 202.
Greenstein (1931). *J. Biol. Chem.* **93**, 479.
—— (1932). *J. Biol. Chem.* **95**, 465.
—— (1933). *J. Biol. Chem.* **101**, 603.
Kirk and Schmidt (1929). *J. Biol. Chem.* **81**, 237.
Levene, Simms and Pfaltz (1926). *J. Biol. Chem.* **70**, 253.
Melville (1935). *Biochem. J.* **29**, 179.
—— and Richardson (1934). *Biochem. J.* **28**, 1565.
von Muralt (1930). *J. Amer. Chem. Soc.* **52**, 3518.
Richardson (1934, 1). *Proc. Roy. Soc. Lond.*, B **115**, 121.
—— (1934, 2). *Proc. Roy. Soc. Lond.*, B **115**, 142.
Simms (1926). *J. Amer. Chem. Soc.* **48**, 1244.
—— (1928). *J. Gen. Physiol.* **11**, 629.
Stiasny and Scotti (1930). *Ber. deutsch. Chem. Ges.* **63**, 2977.

XXV. OBSERVATIONS ON THE NATURE, DISTRIBUTION AND DEVELOPMENT OF CERTAIN CELL-WALL CONSTITUENTS OF PLANTS.

By HAROLD WILLIAM BUSTON.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

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The view has frequently been advanced that the pectic substances of plants, whatever their precise chemical nature, are not to be regarded as fixed and permanent constituents of the cell-wall, but rather as labile substances that may undergo further changes, possibly of very profound nature, leading to their almost complete disappearance from the older tissues. By such transformations of pectin, two classes of substances have been supposed to arise, (i) lignin, (ii) the hemicelluloses. It has in fact been suggested [O'Dwyer, 1926; 1928; 1932] that all three types of compound are interrelated, the passage from pectin to lignin taking place through certain hemicelluloses.

With regard to the former, Fuchs [1927] has advanced a theory of lignification in which he emphasises the fact that pectin and cellulose disappear as lignification sets in—a point previously made by von Fellenberg [1918]. Fuchs sees further a parallel to this development in the individual plant in the apparent development of a more and more modified cell-wall as one passes from the Algae to the higher plants; thus the algal cell-wall (he states) consists of pure cellulose, while in the mosses, incrusting substances (pectins) are present, lignins only appearing at a higher evolutionary level. As conceived by Fuchs, lignification involves the oxidation of the primitive cell-wall to "acid saccharides," and especially to pectin, with subsequent transformation of these, by unknown stages, into lignin. Ehrlich also [1930] considers that the acid polysaccharides form the chief cell-wall material, and that the formation of lignin represents a process involving enzymic dehydration and reduction of these bodies and affecting particularly the pectins. He claims that from flax stems (*i.e.* a partly lignified tissue) it is possible to isolate an "altered pectin," giving rise on hydrolysis to less galacturonic acid than normal pectin and containing also xylose residues in the molecule; with this is associated a brown substance having many of the properties of lignin, giving the characteristic colour tests of the latter, having almost the same empirical composition and containing a similar proportion of methoxyl [Ehrlich and Schubert, 1926; 1928]. This brown substance he regards as a substance derived from the pectin and intermediate between it and lignin.

It has been claimed also that microchemical staining tests show that there is a replacement of pectin by lignin in older tissues [Mehta, 1925], while Ritter [1925] states that 0·5 % ammonium oxalate, a recognised reagent for extraction of pectins, does not remove any pectic substance from basswood, red alder or pinewood (age of wood not stated), and he infers therefore the absence of pectin from woody tissues.

Finally the common possession of a high methoxyl content, both by soluble pectin and insoluble lignin (at least, as isolated), has been regarded as evidence of their interrelation. Ehrlich [1930] also considers that the hydroxyl and acetyl groups of the lignin molecule represent those present in the parent polysaccharide.

No complete scheme representing the course of the supposed transformation of pectin into lignin seems to have been formulated—indeed, in view of the lack of understanding of the structure of lignin, none is possible as yet—but such changes if taking place must necessarily be of the most profound nature.

A relationship between the pectins and the bodies known as structural hemicelluloses is much more readily understood. The acid saccharides or polyuronides in general comprise three groups of structural units, *viz.* the anhydrides of hexoses, hexuronic acids and pentoses, combined in widely varying proportions. Chemically, the hexoses are considered to represent the parent substances forming the cell-wall material as originally laid down. By oxidation of their free primary alcoholic groups, after condensation of the sugars themselves to hexosans, there would be formed uronic anhydride units, and by subsequent decarboxylation of these, pentosan units—the whole process taking place actually within the cell-wall. Stereochemical considerations show that, by such a process, arabans would arise from galactans, while glucosans would pass through glucuronic anhydrides to xylans. It is evident that from a parent hexosan, even if based on a single sugar, a very wide variety of intermediate products could arise as the result of such transformations; it is thus not surprising that very different substances, all legitimately classed as hemicelluloses or acid saccharides, have been obtained. Among these substances the pectins represent a type of unusually highly oxidised hexosan, with but little of the unchanged parent hexose and little of the ultimate pentose constituent. Pectins thus are mainly distinguished from the hemicelluloses in that they contain some 70 % of uronic anhydride, whereas in the hemicelluloses the figure rarely exceeds 25 %.

Support has been found for the suggestion that the pectins are the precursors of the hemicelluloses in the frequently-repeated observation that the former predominate in young, fleshy or unlignified tissues, while in older lignified tissues, of lower moisture content, the position is reversed. From the more purely chemical side, the *in vitro* transformation of pectins into hemicellulose-like bodies has been reported by Candlin and Schryver [1928] and Linggood [1930] who used a method involving decarboxylation by heating under pressure; and by Norman and Norris [1930] who employed a process of mild oxidation.

It is evident therefore that, taken as a whole, the evidence for the existence of a relationship between the three groups of substances is considerable. All these suggestions, however, need some further examination. In the first place there seems to be no record of any investigation dealing with the distribution of pectin, hemicelluloses and lignin, and possibly other allied carbohydrates, in a given plant or tissue during its life-period, the statements concerning the relative amounts of these substances present in young or old material being based rather upon isolated observations on different types of material. Thus speculation has been founded upon data concerning the relative proportions of the different groups of constituents, rather than concerning the absolute amounts of the substances present in a plant from time to time. It is by no means impossible that observations which have been interpreted as indicating the transformation of one substance into another really indicate a changing course of metabolism in the plant, giving rise to increasing amounts of a new type of product, while formation of the original type ceases—that already present remaining intact, however. Preece [1930], for instance, records the presence of 0·4 % of pectin in boxwood, while O'Dwyer [1925] actually isolated a small amount of pectin from beechwood 80 years old. Although these relative proportions are low, the total weight of pectin in the whole plant must have been considerable. If pectin were formed only in the early stages of growth, while subsequent thickening of the cell-wall involved the laying down of large amounts of non-pectin material, the low concentration of the pectin would be accounted for, without the necessity for postulating any extensive subsequent alteration of pectin.

Concerning the chemical work mentioned above, the products obtained by the various investigators certainly had a much lower uronic anhydride content than the original pectins, but still considerably higher than met with in any of the hemicelluloses extracted from plant materials, with the sole exception of O'Dwyer's beech hemicellulose B [1926] which contained some 60 % of uronic anhydride, a figure not approached in any other hemicellulose recorded. Again the yields of the products were generally very low (Candlin and Schryver [1928] report a yield of some 15 %), and in some cases may have consisted in part at least of hemicelluloses present as impurity in the original pectin. The nature of the sugar residues in these products is not recorded.

As regards the evidence based upon microchemical staining tests this must be accepted with caution. The extremely complex nature of the cell-wall, the constituents of which may be present in the form of an intricate mosaic, and not differentiated into sharply divided layers, and the admitted lack of specificity of many of the stains used render the evidence from this direction of doubtful value, at least when taken alone.

Thus it will be seen that the evidence for the transformation of pectin into hemicellulose and/or lignin *in vivo* is by no means conclusive, although perhaps, in the mass, impressive. It seemed therefore appropriate to attempt experiments which would yield more direct evidence of the occurrence or possibility of the transformations suggested. Since the ready lability of the pectins is assumed in all the theories outlined above, attention was in the first place directed to these substances, and experiments were made with the object of ascertaining whether such assumptions are justified. At the same time, the investigations furnished much useful information in other directions. For instance, a search through the literature revealed the paucity of direct information concerning the cell-wall constituents of the leaf, and certain particulars concerning these bodies came to light during the course of the work. Again, observations were made concerning the availability of some of the supposed cell-wall materials as reserve carbohydrate for purposes of metabolism.

Concerning the methods of investigation which seemed suitable, the most obvious consisted in following out the distribution of the various substances under consideration in a given plant during its full period of development. To carry out thoroughly such an investigation on a typical woody plant would obviously be a matter of considerable difficulty. On the other hand, it is possible to select a part of a plant which in the course of a season's growth will show an alteration in character somewhat resembling that met with in the development of woody tissues as a whole. Data of this type may be found in the rather isolated observations published by Schulze and Pfenniger [1910] regarding the hemicellulose content of the pods of garden peas and beans. In the case of beans particularly, the young pods are of fleshy nature, with a high water content, a state regarded as quite typical of tissues in which pectins predominate; while with maturity the water content decreases enormously, and the tissues become hardened and fibrous, resembling externally at least tissues in which much hemicellulose may be found. Staining tests also show that some lignin-like material has developed in the final stages of ripening. Schulze and Pfenniger found that the proportions of hemicellulose in the pods increased considerably with maturity.

No figures for pectin content are given by these authors—in fact, the methods for estimation of these substances had not then been established. Further, Schulze and Pfenniger estimated their hemicellulose by a method of difference, and therefore possibly included certain other substances under this heading. In the present work, the cell-wall constituents of runner bean pods and the pods of sweet peas were investigated.

Although as regards steadily diminishing water content these tissues resemble to some extent those parts of the plant undergoing lignification, yet they cannot be considered as typical lignified materials, and at the most the changes taking place during their ripening could only be expected to simulate the very early stages of lignification. It was therefore desirable to carry out a similar investigation dealing with the growth of a true woody tissue. Suitable material may be found in the hard-wooded shrubs. For instance, roses normally throw up, in the spring, strong growths from the base of the plant, whose early growth is extremely rapid, and which are very definitely of a fleshy nature. During the summer and autumn these shoots become "ripened," *i.e.* lose their water slowly and undergo true lignification. Opportunity arose to conduct a (single) controlled experiment upon such growths, as described below.

Apart from such direct studies as those outlined above, artificial conditions may be set up which will tend to promote transformation of cell-wall materials where such is possible. To this end examination was made of detached leaves, (i) after a period of "starvation" in darkness, (ii) after allowing the leaves to undergo a process of slow drying in sunlight. The first-mentioned process was carried out by allowing the detached leaves to remain, with their petioles in water, in darkness for varying periods of time (7 days and upwards) until, in extreme cases, visible wilting had taken place. Under these circumstances, photosynthesis ceases, respiration continues, and there is an ample water supply. Thus it is to be expected that, after exhaustion (by respiration) of the readily available sugars, there might be some oxidation of reserve polysaccharides, either directly or after hydrolysis to simple sugars. On these lines, if decarboxylation of uronides is a process of mild oxidation, as suggested by Norman and Norris [1930], then prolonged starvation might possibly lead to such decarboxylation, the pectins with their high uronic anhydride content reflecting this process. Some early experiments on these lines were recorded by Deleano [1912] and by Ahrns [1924]. The former subjected leaves of *Vitis* to starvation for periods up to 21 days, but observed breakdown only of simple sugars and of starch; no figures for pectin were recorded. Ahrns, using much less drastic methods, obtained somewhat corresponding results.

The second process, that of slow drying, may be regarded as somewhat analogous to the conversion of grass into hay. In this case, both photosynthesis and respiration might be expected to continue during the earliest stages of the process, but would rapidly be terminated by depletion of water-supply. Thus respiration would probably be too short-lived to occasion any mobilisation of possible reserve carbohydrate; the diminished water supply would, in fact, be the most important factor altering the course of metabolism. As Spoehr and McDougal [1918] and McDougal *et al.* [1919] have shown, a depleted water supply induces a conversion of saccharides into anhydrides and leads to an accumulation of pentosans. The thickening of tissues (*e.g.* during lignification) is accompanied by a progressive decrease in their water content, a factor which may well influence the building up of the cell-wall or the modification of its constituents. If the pectins become converted with increasing age into hemicelluloses, and possibly lignin, then it might be expected that the same process would be encouraged by the gradual withdrawal of water from the tissue by slow drying.

Apart from the above-discussed suggestion, regarding interconversion of cell-wall constituents, the statement has frequently been made that pectins are formed in tissues during periods of rapid growth, hemicelluloses arising with less rapid development. It seemed therefore appropriate to examine the distribution of these cell-wall constituents in plants of the same variety grown under conditions that would produce different rates of growth, *i.e.* plants grown normally and "forced." A brief experiment of this type is recorded.

The various lines of attack outlined would in the sum be expected to yield information of the kind sought and at the same time to furnish useful data in the other connections mentioned. The isolation of the different types of hemicellulose from leaves has not been recorded hitherto. Some of these substances were of necessity handled in the present research, and information regarding their chemical nature is recorded.

Apart from the difficulty of providing suitable material (*e.g.* for any prolonged series of investigations on the development of lignin in a typical woody plant), there are considerable difficulties of analysis to contend with. While for instance the calcium pectate method for determination of pectin may be regarded as satisfactory for fruits *etc.*, where extraction is easy, the physical nature of wood might make thorough extraction much more difficult, although pectins existed in the tissue. Again the methods of estimation of the hemicelluloses so far available are far from satisfactory. The term, as usually applied, and as used in the present work, indicates those constituents not extracted by water, but removed by extraction with 4 % hydroxide solution. (Their solubility after extraction frequently alters). Thus certain substances, whose chemical nature would warrant their inclusion in the group, would be excluded; among such would be the water-soluble galactans of wood [Schorger and Smith, 1916], of fruits [Widdowson, 1931] and the unextracted xylan found in close association with cellulose and called by Hawley and Norman [1932] "cellulosan."

EXPERIMENTAL.

1. *General analytical methods.*

Since the investigation involved a very large number of routine analyses, it will save repetition if the methods usually employed are summarised in the first place, reference to any special modifications being made in dealing with the individual experiments.

(*a*) *Preparation of material.* In the case of leaves, these were dried in a large steam-heated oven through which a current of air was drawn; drying was carried out as soon after picking the leaves, and as rapidly, as possible. The material was usually allowed to remain in the oven for 4–5 hours and was then transferred to stoppered glass jars, cooled, weighed and ground finely. Before using, the material was passed through a sieve, and the portion of 30–60 mesh size (per inch) taken for experiment; this was dried overnight in an oven before being weighed out for extraction.

In some cases (noted below) drying was carried out by dropping the material into six times its weight of boiling 95 % alcohol; after boiling for a sufficient time, the material was transferred to the steam-oven and the process completed as described.

(*b*) *Estimation of pectin.* Suitable samples (1–5 g.) of the dried material were extracted with 0·5 % ammonium oxalate solution in a water-bath at 95° for

6 hours; the residues were treated twice more in the same manner. In the extract, pectin was estimated as calcium pectate, following a preliminary precipitation with acidified alcohol, according to the method of Emmett and Carré [1926]. An amount of extract was taken sufficient to provide 0·02–0·04 g. of calcium pectate for weighing.

(*c*) *Estimation of hemicelluloses.* Hemicelluloses were estimated by a method similar to that of Preece [1930], and already described in detail by the present author [1934]. According to this method, the material, after removal of pectin, was treated once with dilute alcoholic sodium hydroxide (1 % NaOH in 50 % alcohol), and then exhaustively extracted with 4 % sodium hydroxide. The hemicellulose precipitated by acidification and addition of excess of alcohol was, after suitable washing, weighed directly on a Gooch crucible. Differentiation into "hemicellulose A" (pptd. on acidification only) and "hemicellulose B *plus* C" (requiring excess of alcohol for pptn.) was made where desirable. No separation of the fractions B and C was attempted. In all cases corrections for ash and for protein present were made, as suggested by Preece. Amounts of material were chosen to give 0·15–0·30 g. of hemicellulose for weighing.

(*d*) *Other estimations.* Lignin was estimated by the method of digestion with 72 % sulphuric acid, according to the details given by Dorée [1933]. Cellulose was estimated by the Cross and Bevan chlorination technique. Estimations of furfuraldehyde were made by the usual distillation method, the furfuraldehyde being weighed as phloroglucide, while uronic anhydride was determined where necessary by the method of Nanji *et al.* [1925].

In the cases of the pectin and hemicellulose estimations, duplicate estimations were in all cases performed upon aliquot parts of the extracts and, except in a few cases where lack of material prevented, duplicate extractions were carried out. The other estimations were normally carried out in duplicate.

2. *The distribution of cell-wall materials in pods of beans and sweet peas during development.*

The material used was obtained from the open garden during the summers of 1933 and 1934, no special cultivation being made. In the case of the bean pods, all were picked at the same time and graded according to size in five or six batches, this being regarded as sufficient guide to their stage of development. In the case of the sweet pea pods, dated labels were attached to the separate flowers as the petals faded, so that the actual age of the pods themselves, from the time of forming, was recorded.

Drying of the material was carried out by the alcohol method, after removal of the seeds, only the shells being used for experiment. The figures given in Table I for percentage content of pectin *etc.* are calculated on the dry weight of material after alcohol treatment; values are also given for actual weight of the constituents per 100 pods.

The results in all cases showed a steady development of both pectin and hemicellulose throughout the whole period of the experiment. The very young pods contained relatively less pectin than the older, in which both pectins and hemicelluloses remained at a fairly constant level; both types of substance seemed therefore to be produced throughout the whole period of development. It was of interest to note the very different ratios of pectin to hemicellulose in the two types of tissue; the pods of the sweet pea are relatively of a much drier nature than those of the bean, especially in their earlier stages, and the preponderance of hemicellulose therein is in accordance with common experience.

Table I. *Distribution of pectins* etc. *in pods of* Phaseolus *and* Lathyrus.

Series I. *Phaseolus.* Tall variety, grown at Chelsea, 1933.
Picked 15. viii. 1933.

Batch No.	1	2	3	4	5	6
Average length of pods when picked (cm.)	2·5	5·0	7·5	10·0	15·0	21·0
Wt. of alcohol-dry residues per 100 pods (g.)	3·82	7·55	17·9	27·7	69·0	99·2
Pectin, as calcium pectate, % of dry weight	9·8	9·5	10·3	14·1	—	15·0
Hemicellulose, % of dry wt.	(17·2*)		18·9	16·3	19·0	18·3
Pectin, g. per 100 pods	0·37	0·72	1·68	3·52	—	14·9
Hemicellulose, g. per 100 pods	(0·98*)		3·35	4·50	13·1	18·2

Series II. *Phaseolus.* Dwarf variety, "Little Gem"; grown at Outwood, Surrey, 1934.
Picked 19. x. 1934.

Batch No.	1	2	3	4	5
Average length of pods (cm.)	4	7	9	11	13
Wt. of alcohol-dry residues per 100 pods (g.)	4·55	7·84	29·8	35·6	71·0
Pectin, as calcium pectate, % of dry wt.	11·1	11·4	14·4	15·4	13·1
Hemicellulose, % of dry wt.	14·0	13·8	13·1	16·4	17·3
Pectin, g. per 100 pods	0·50	0·89	4·29	5·49	9·3
Hemicellulose, g. per 100 pods	0·64	1·07	3·90	5·84	12·3

Series III. *Lathyrus.* Grown at Outwood, Surrey, 1933.

Batch No.	1	2	3	4
Age when picked (days)	3	8	16	23
Wt. per 100 pods (g.)	2·2	7·4	12·1	23·2
Pectin, as Ca pectate, % of dry wt.	2·63	3·75	4·26	4·31
Hemicellulose, % dry wt.	29·2†	26·0	27·7	22·25
Pectin, g. per 100 pods	0·06	0·28	0·52	0·99
Hemicellulose, g. per 100 pods	0·64†	1·94	3·35	5·11

* Average of batches 1 and 2; insufficient material for separate determinations.
† One extraction only.

The values for hemicellulose content of the bean pods are in fairly good agreement with those of Schulze and Pfenniger [1910].

This investigation was extended to include examination of fully grown bean pods in both the green and the brown (fully ripened) conditions, in order to see whether the pectin content was altered in the final stages of development. Micro-chemical examination of brown pods showed that they contained some amount of thickened tissue, giving the reactions of lignin; in this state the pods were almost completely "air-dry." Green and brown pods of the same size were chosen, assuming that equivalent development had taken place in each case.

In the 1934 series, the "brown" pods were of two types, (i) allowed to remain on the plants while ripening, and (ii) detached when fully grown, but still green, and allowed to become brown by remaining in sunlight under glass (*cf.* the process of slow drying described below, p. 207). After removal of the seeds, drying, sampling *etc.* were carried out as usual (see Table II).

From these figures it seemed evident that the pectins remained unaffected even at the end of tissue development; in the 1933 series there was even a small increase of pectin at the end of the period—possibly owing to the "green" pods having been gathered rather before they had fully developed. Complete ripening resulted in a small loss of total dry matter in each case and in a definite loss of hemicellulose. It may be pointed out that in the case of the sweet peas, batch no. 4, the pods were turning brown, and a relative decrease in hemicellulose was to be observed there also. According to Schulze and Pfenniger, part of the hemicellulose is withdrawn from the pods during the later stages of ripening and translocated to the seeds themselves. It is interesting to compare certain of the

Table II. *Distribution of pectins* etc. *in mature bean pods.*

Material	Outwood, 1933 (Dwarf type, short pods)		Chelsea, 1934 (Tall type, long pods)		
Ripened	— Green	On plant Brown	— Green	On plant Brown	Detached Brown
Dry wt. of material insol. 80 % alc., per 100 pods (g.)	62·5	56·6	109·0	132·0*	92·0
Pectin, as Ca pectate, % of dry material	12·6	15·0	17·8	14·5*	21·2
Hemicellulose, % of dry material	14·7	13·2	21·4	12·5*	17·9
Lignin, % of dry material	13·2	14·9	8·5	9·4*	—
Pectin, g. per 100 pods	7·99	8·49	19·4	19·1	19·5
Hemicellulose, g. per 100 pods	9·17	7·60	21·1	16·5	18·0
Lignin, g. per 100 pods	8·25	8·43	9·26	12·4	—

* Material not dried by alcohol. (See also Table V, p. 206.)

figures for corresponding batches grown in the two seasons, 1933 and 1934, *viz.* Series II, batch 5, of Table I, with "Outwood, 1933, green" (Table II); and Series I, batch 6, Table I, with "Chelsea, 1934, green" (Table II). In each case, total growth is greater in the 1934 batch, probably owing to the greater rainfall during the growing season; and at the same time, the relative proportions of both pectins and hemicellulose are higher. This point is further commented upon below (p. 214).

Among the other figures given, attention may be called to the small increase in lignin in the 1933 series and the very definite increase in the pods ripened on the plant in 1934.

3. *Changes in the cell-wall constituents of wood of rose during lignification.*

A vigorous hybrid tea rose ("Leni Neuss") was pruned severely in April 1934; it was noted that within a few weeks two new shoots had appeared from a dormant bud on one stem, and were making rapid growth. Their rates of growth were similar, and a terminal bud appeared on each at the end of June. On July 4th the length of each was 53 cm., with 13 internodes; it seemed therefore that the two shoots might be regarded as identical at this stage. From each shoot the apical bud and side leaves were removed. One shoot was cut off, 5 cm. from its base (*i.e.* at the lowest node) on that date. The second shoot was allowed to remain on the plant, any fresh growths from axillary buds being removed immediately, until October 24th. Shoot no. I when removed was mainly of fleshy growth, although the lower part had already become considerably hardened. During the warm dry summer of 1934 the second shoot had become thoroughly "ripened," and was hard and woody throughout its length. A small increase in length (measured from the first node) had occurred, and the water content of the shoot was much less (Table III).

Each shoot on removal was sliced thinly and dried by the alcohol method, and the necessary analyses were performed, the results of which are shown in Table III. In the calculations of the percentage composition, allowance was made for the quantity of material removed in the alcoholic extracts. From shoot no. I only single extractions were possible; the cellulose determination was carried out on the residues from the extraction of hemicellulose.

From these results it appeared that formation of pectin continued throughout the whole period of development, the percentage showing however a decrease in the older shoot. In contrast, there was a much greater development of hemicelluloses, and still more so of lignin, the total amount of which in the shoot increased six times. The significance of these results is further considered below (pp. 212, 216).

Table III. *Distribution of cell-wall constituents in woody shoots of rose.*

	Shoot I	Shoot II
Age when cut (weeks)	12	28
Length (cm.) when cut	48	55
Fresh wt. when cut (g.)	27·0	27·5
Total dry wt. (g.)	4·06	10·51
Pectin, % dry wt.	5·03	4·77
Hemicellulose, % dry wt.	13·46	18·93
Cellulose (C and B), % dry wt.	24·0	26·3
Lignin, % dry wt.	7·94	18·36
Total pectin in shoot (g.)	0·204	0·49
Total hemicellulose in shoot (g.)	0·55	0·99
Total cellulose in shoot (g.)	0·97	2·76
Total lignin in shoot (g.)	0·33	1·93

4. *Changes in cell-wall constituents of leaves during "starvation*[1]."

Three types of material were used in these experiments: (*a*) leaves of runner-bean, (*b*) leaves of vine, (*c*) laminae of maize. Picking was in all cases carried out in the early morning, when but little starch was present. The batches of leaves were divided into "control" and "experimental" lots, care being taken to obtain fair average samples in every case. The control leaves were dried immediately in the oven, after removal of their petioles; the experimental leaves were placed with their petioles in jars of water and kept in darkness throughout the experimental period. The water in the jars was changed daily, and on each occasion it was found desirable to remove a small portion of the tip of the petiole. At the end of the period, the petioles were removed, and the laminae dried under the same conditions as the controls. In the case of the maize plants, a somewhat different procedure was necessary; whole plants of as nearly the same size as possible, were chosen, and were usually found to be of almost the same fresh weight (within 5 g. on a weight of 75–100 g.). The lower ends of the stalks were sufficiently trimmed to equalise the weights of control and experimental batches (4 whole plants in each batch), and the complete plants were treated, *i.e.* starved, dried *etc.* It was noted that both vine and maize were far more resistant to the starvation process than was the bean; in one experiment maize plants remained for 45 days without any appreciable wilting. Further, in the case of maize, the loss of dry matter during starvation was comparatively little. Table IV summarises the results of these experiments.

It was evident from these figures that the pectins remained practically unaffected by the process of starvation. In only one case was any appreciable loss of pectin noticed; in one experiment on the bean leaves. The loss of 3·6 % of the total pectin, here recorded, was probably outside the limits of experimental error; and it is perhaps noteworthy that in this case, the only one in which loss of pectin could be suspected, there was an extreme loss of total material. (In the case of maize, 1933, the total pectin was so low that the differences in amount between the two batches are of doubtful significance.) The leaves used in this test were younger than in the other cases, and contained more water-soluble matter, a fact which may have accounted for the unusually great loss of material.

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[1] Many of the analyses recorded in this section were carried out by Dr V. H. Chambers, who was responsible also for the preparation of the material (bean, 1932 batches, and vine).

Table IV. *Effect of starvation on cell-wall constituents of leaves.*

All values (pectin *etc.*) are given as % on dry weight of original tissue.

Material	% loss of solids	Pectin (as Ca pectate)	% change of pectin	Hemi-cellulose "A"	% change	Hemi-celluloses "B"-"C"	% change	Hemi-cellulose total	% loss
Bean, 1932, I									
Control	—	6·55	—	1·09	—	2·14	—	3·23*	—
Starved 7 days	31	6·31	−3·6	0·97	−11·0	1·32	−38	2·29*	−29
Bean, 1932, II									
Control	—	7·74	—	1·06	—	1·59	—	2·65*	—
Starved 7 days	17·5	7·92	+2·3	1·09	+2·8	1·23	−23	2·32*	12
Bean, 1933									
Control	—	8·96	—	1·77	—	5·22	—	6·99*	
Starved 10 days	21	9·03	+0·8	1·78	+0·6	3·22	−38	5·00*	−28
Vine, 1932									
Control	—	6·90	—	1·84	—	2·50		4·34*	—
Starved 21 days	27	6·75	−2·2	1·92	+4·3	0·84	−66	2·76*	−36
Maize, 1933									
Control	—	0·90	—		—		—	25·5	—
Starved 46 days	6	1·04	(+15)	—		—	—	24·5	− 4
Maize, 1934									
Control	—	1·39	—	10·9*	—	14·7	—	25·6	—
Starved 19 days	10	1·43	+2·8	11·4*	+1·6	11·9	−19	23·3	− 9

* Values calculated, by sum or difference; other values by direct estimation.

Turning to the figures for the hemicelluloses, these in all cases showed distinct losses during the starvation period, but, except in the case of the younger bean leaves, the loss was invariably confined to the water-soluble hemicelluloses (fractions "B" and "C"), where the loss in one case reached 66 % of the total fraction.

The nature of the individual hemicellulose fractions was examined in most cases (see p. 211) and determinations of pentosan and uronic anhydride were carried out. The insoluble hemicelluloses ("A") of the bean and vine leaves proved to be hexosans, no more than a trace of furfuraldehyde being evolved on boiling with 12 % HCl. On the other hand, the soluble ("B"-"C") fractions were found to contain units of pentosan and uronic anhydride. The only other recorded examples of hemicelluloses from leaf material were those of grass [Buston, 1934]. Of these, "A" was found to be composed almost entirely of xylan, giving a furfuraldehyde yield of 55 % approximately, while the "B" and "C" fractions contained a variety of pentosans, hexosans and uronic anhydrides. From maize, the "A" fraction again contained much pentosan, while the other fractions also closely resembled those of grass. Further details of these products are recorded in Table IX.

The soluble hemicelluloses isolated from the leaves after starvation were found to differ considerably from those of the control groups, as judged by furfuraldehyde determinations, the results of which are summarised in Table V. In general there was a very considerable loss of hexosan, together with some increase of uronic anhydride. Some net loss of pentosan also seemed to have occurred, so that the difference was perhaps not due simply to loss of hexose sugar by respiration. In Table V are also included some observations on the changes shown by the hemicelluloses of the bean pods during the final stages of ripening. Here again there is a striking decrease in the hexosan content, probably owing, as suggested by Schulze and Pfenniger, to translocation to the seeds, although possibly partly due also to respiration.

The stability of hemicellulose "A" in the bean and the vine leaves seemed curious. This substance, as stated above, was of hexosan type, so that it might

Table V. *Changes in constitution of hemicelluloses of leaves during starvation.*

Material	Sugars in hydrolysis mixture	Total furfuraldehyde (%)	Uronic anhydride (%)	Pentosan (%)	Hexosan (%)
Bean leaf, 1933, control					
Hemicellulose "A"	Galactose	0	Trace	0	>90·0
Hemicelluloses "B"-"C"	Galactose, arabinose, galacturonic acid	18·4	19·6	22·4	58·0
Bean leaf, 1933, starved	Not examined—assumed to be as above				
Hemicellulose "A"		0	0	0	>90·0
Hemicelluloses "B"-"C"		22·2	29·6	24·5	45·9
Bean leaf, 1932, I					
Control "B"-"C"		18·7		Not determined	
Starved "B"-"C"		24·9		,, ,,	
Maize, 1933, control Total hemicellulose	Xylose ? uronic acid ?hexose	45·7	(<5)	Not determined	
Maize, 1933, starved Total hemicellulose		48·2	(5–7)	,, ,,	
Bean pod, 1933	Galactose, arabinose, galacturonic acid				
Green Total hemicellulose		21·0	11·2	30·1	58·7
Brown Total hemicellulose		33·9	12·6	50·8	36·6

Table V*a*. *Net amounts of pentosans, uronic anhydride and hexosan in the hemicellulose fractions of leaves*, etc.

	Net wt. of saccharide constituent (g. per 100 g. dry leaf)		
Material	Pentosan of hemicelluloses "B"-"C"	Uronic anhydride of hemicelluloses "B"-"C"	Hexosan of hemicelluloses "B"-"C"
Bean leaf, 1933			
Control	1·17	1·02	3·03
Starved	0·79	0·95	1·48

	Net wt. of saccharide constituent (g. per 100 pods)		
	Pentosan in total hemicellulose	Uronic anhydride in total hemicellulose	Hexosan in total hemicellulose
Bean pod, 1933, fully-grown			
Green	2·75	1·03	5·39
Brown	3·86	0·96	2·78

have been expected to be open to metabolic change. It was found to yield on hydrolysis, galactose only—not a readily oxidised sugar. On the other hand, no hexose other than galactose could be detected in any of the hemicelluloses, even those which had undoubtedly undergone some change.

It was observed that the hemicelluloses isolated from the leaves of the bean and the vine (fraction "A"), but not of the maize, were always contaminated with considerable amounts of protein, as much as 30 % of the fraction in some cases being of protein nature (as judged by its nitrogen content). The contamination was much less in the case of the starved leaves, and the other hemicelluloses ("B"-"C") were free from protein. It will be recalled that Chibnall [1924] has shown the disappearance of protein from leaves subjected to the starvation process.

Some difficulty was experienced in dealing with the vine hemicelluloses. All the vine extracts were extremely dark in colour and inclined to be viscous, and attempts to estimate the "A" and "B"-"C" fractions separately by the ordinary method were unsuccessful. An estimate of the relative amount of each present was obtained by determining the furfuraldehyde yield of the whole fraction (mixed), and of a small portion of the "B"-"C" fraction isolated from a further batch of material; from these values, knowing that fraction "A" gave no furfuraldehyde, it was possible to calculate the relative amounts of the two types. The vine leaf is known to contain large quantities of organic acids, and the presence of these substances, or some of their degradation products, may have led to an alteration in the physical nature of the hemicelluloses, causing the difficulty. It may be mentioned further that although no difficulty was met with as a rule in identifying the sugars in the hydrolysis mixtures, with the vine products it is possible that some sugars have escaped identification, as the yields of the different derivatives were poor and crystallisation thereof always slow.

In the case of maize, the loss of material was apparently confined to the water-soluble hemicelluloses. Since however in this case the "A" fraction was mainly xylan, it was hardly to be expected that this would show any alteration. Some change in its physical condition had occurred, since it was much less readily precipitated on acidification of the extracts from the starved leaves.

5. *Changes in the cell-wall constituents of leaves during a process of slow drying.*

Leaves of the bean and the vine were again used, the method of sampling being identical with that adopted in the starvation experiments. Controls were dried in the oven, while the experimental leaves were spread on paper in an ordinary garden frame, the lights of which were removed during fine weather but replaced at night. The leaves were allowed to dry in this manner for 7–10 days, after which they were shaken to remove dust, and drying was completed as usual. Estimations of pectin and of hemicelluloses were carried out as in the previous experiment (see Table VI). Again the pectin remained unchanged by the treatment. The hemicelluloses had undergone modification, but in a manner different from that met with in the starvation experiments. In each case there

Table VI. *Effect of slow drying on cell-wall constituents of leaves.*

Values expressed as % of dry wt. of original tissue.

Material	Pectin (as Ca pectate)	Hemicellulose "A"	% change	Hemicellulose "B"-"C"	% change	Hemicellulose total	% change
Bean, 1932, I							
Control	6·55	1·09	—	2·14	—	3·23†	
Dried	6·50	2·47	+125	1·72	−20	4·19†	+30
Bean leaf, 1932, II							
Control	7·74	1·06	—	1·59	—	2·65†	
Dried	7·71	N.d.*	—	N.d.	—	3·57	+35
Bean leaf, 1933							
Control	N.d.	1·77	—	5·22	—	6·99†	
Dried	N.d.	2·75	+56	3·51	−33	6·26†	−10
Vine leaf, 1932							
Control	6·96	1·23	—	3·66	—	4·89†	
Dried	7·31	2·92	+137	2·92	−20	5·84†	+19

* Not determined. † By addition.

was a considerable increase in the insoluble fraction ("A"). Its furfuraldehyde yield however remained *nil*, so that there was no evidence of any production of pentosan. Definite loss had occurred in the soluble hemicelluloses, and again an increase in the proportion of pentosan was observed (see Table VI*a*). Total hemicellulose increased in some cases, and the increase was probably due to anhydride formation from hexoses, or simpler soluble hexosans, during the gradual withdrawal of water. Whether the disappearance of some of the soluble hemicelluloses was due to loss by respiration during the early stages of the treatment (which seems hardly likely) or to some alteration in their physical condition could not be judged.

Table VI*a*. *Changes in constitution of hemicelluloses on drying of leaves.*

	Furfuraldehyde yield %		Furfuraldehyde yield %
Bean leaf, 1932, I Hemicelluloses "B"-"C"		Bean leaf, 1933 Hemicelluloses "B"-"C"	
Control	18·7	Control	18·4
Dried	23·7	Dried	23·2

No experiment was made with maize in this case, but examination was made of a sample of hay prepared from a grass previously examined. Some analyses of this grass have already been recorded [Buston, 1934]. The grass (cocksfoot) was fully grown when cut, but still green. The crop from one half of the plot was oven-dried as soon after cutting as possible; the remainder was cut at the same time, spread and raked in the usual manner until quite brown and then oven-dried. This process of hay-making was thus somewhat similar to the slow drying process carried out with the bean and vine leaves. In view of the economic importance of hay-making, it is strange that so little attention has been paid to the chemistry of the process. Fleischmann [1912] records the observation that "crude fibre" remains unchanged, and that there may be a small increase in total dry matter, but little further information seems to have been published. A rather more extended study was therefore made of the cocksfoot hay, the results of which are summarised in Table VII. Unfortunately the figures obtained could not be calculated to "dry weight of original grass," since the relative weights of grass and hay were not determined; values are given as % of dry material, grass or hay. Thus any loss or gain in dry weight by respiration or assimilation is unaccounted for; it may be pointed out, however, that the ash

Table VII. *Comparison of constitutions of grass and hay.*

All figures given as % dry weight of grass or hay.

	Ash	Water soluble material	Reducing substances as glucose	Reducing substances after hydrolysis	Pectin as Ca pectate
Grass	8·16	21·9	3·54	5·57	1·97
Hay	8·14	19·5	4·95	5·68	1·95

	OMe in pectin* %	% total pectin as pectate	Total hemicellulose	Cross & Bevan cellulose	Xylan in cellulose	Lignin
Grass	2·8	76	18·31	33·2	8·5	16·85
Hay	1·3	92	18·47	33·2	N.d.	17·05

* Pectin as isolated from large-scale ammonium oxalate extracts.

contents of the two samples were almost identical, so that any considerable loss or gain of dry matter during conversion into hay was not indicated. The methods of analysis (*e.g.* for soluble carbohydrates *etc.*) were those given in the previous paper [1934]. The results showed that the cell-wall material remained practically unchanged, any variation having been confined to the simpler saccharides.

In hay-making, as carried out according to tradition, considerable variations may be expected in the product, depending mainly upon weather conditions during the harvest. It is evident that the rapid drying during hot weather would give a product very different from that obtained when hay-making is carried out under cool or wet conditions. In the present case not only was the weather extremely hot (summer 1933), but the grass crop itself was of unusually low moisture content when cut. Complete drying must therefore have been very rapid, leaving but little opportunity for alteration in the more stable cell-wall components.

6. *Relative distribution of pectins and hemicelluloses in the radish under varying conditions of growth*[1].

In order to examine the relative distribution of the pectins and hemicelluloses in plant tissues of the same type grown under conditions resulting in different rates of development, the radish was chosen on account of its short period of growth and ease of handling. The seed was sown in rows, one foot apart, and in 8–10 days the seedlings were thinned to 3 in. apart. After thinning, alternate rows in the bed were covered with cloches and the plants grown on for 25 days. At the end of this time the plants were carefully pulled and the roots washed free from soil. The "root" portion, including the swollen hypocotyl, was detached and treated separately from the shoot. A second sowing was made somewhat later in the year and grown in the same manner. It was found actually in each case that the total weight of the plants grown in the open was greater than that of the cloche-grown plants; possibly the latter suffered from lack of moisture, or the temperature inside the cloches was too high. The shoots were oven-dried and the usual estimations performed; the fleshy roots were dried by the alcohol method, and allowance was made for loss of alcohol-soluble material in calculating the results of the determinations of the pectin *etc.* (Table VIII). In considering these figures it is to be noted that the data for the batches cannot be compared directly, since the definitely higher temperature during the growth period of the second batch would no doubt have influenced the type of growth.

Broadly speaking, it seemed that a more rapid growth, as measured by the formation of dry matter over a definite period and taking the plant as a whole, favoured the formation of pectin in an increased ratio to hemicellulose, although in absolute quantities both classes of substance showed an increase with more rapid growth. In batch I the total dry solid formed was almost the same with both normal and cloche-grown plants, and little variation in the pectin/hemicellulose ratio was observed. A wide difference was found, however, in the growth rates of batch II, and here the pectin/hemicellulose ratio showed a distinct difference. The shoots, considered separately, showed a similar result. With the roots, considered alone, the greatest discrepancies were to be observed. The formation of polysaccharides in the root, however, differs from that in the leaf in that the process is dependent upon a supply of sugars translocated downward from the shoot, and therefore only represents the final stage of a process having its earlier stages in another part of the plant. Polysaccharide formation actually observed in the root will therefore be influenced by conditions obtaining

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[1] The experimental work described in this section was largely carried out by Mr H. Thring.

Table VIII. *Rate of growth and formation of pectin* etc. *in the radish.*

Batch I, grown at Chelsea, May 1933; 49 plants in each series.
Batch II, grown at Chelsea, July 1933; 74 plants in each series.

	Dry wt. g.	Pectin % dry wt.	Total pectin formed g.	Hemi-cellulose % dry wt.	Total hemi-cellulose g.	Ratio pectin/hemi-cellulose
A. *Whole plants.*						
Batch I, normal	32·7	10·30	3·37	5·56	1·82	1·85
„ cloche	28·8	11·25	3·24	6·08	1·75	1·85
Batch II, normal	67·2	11·84	7·96	5·80	3·90	2·04
„ cloche	28·6	9·58	2·74	6·01	1·72	1·60
B. *Shoots only.*						
Batch I, normal	24·5	10·65	2·60	5·97	1·46	1·78
„ cloche	24·5	11·47	2·81	6·37	1·56	1·80
Batch II, normal	47·4	13·56	6·43	6·12	2·89	2·22
„ cloche	25·1	9·60	2·41	5·86	1·47	1·64
C. *Roots only.*						
Batch I, normal	8·2	9·45	0·77	4·41	0·36	2·14
„ cloche	4·3	9·92	0·43	4·35	0·19	2·28
Batch II, normal	19·8	7·75	1·53	5·08	1·01	1·53
„ cloche	3·5	9·50	0·33	7·09	0·25	1·32

elsewhere. It seemed therefore best to consider the plants as a whole and to compare the relative formation of the cell-wall constituents in the complete plants. When this is done, the results give support to the suggestion that pectin is formed preferentially during more rapid growth.

7. *Notes on the nature of the hemicelluloses of leaves* etc.

Reference has already been made to the apparent hexose and pentose contents of certain of the hemicelluloses isolated in the course of these experiments. Since these leaf-hemicelluloses do not appear to have been described hitherto, it was thought desirable to identify their constituents where possible. The usual methods for identification of the sugars *etc.* in the hydrolysis mixtures, as given by Browne [1912], van der Haar [1920] and others in the literature, were employed, and the results are tabulated for the sake of brevity. An important difference, as mentioned above, was noted between the hemicellulose "A" of bean, vine *etc.* and that of grass and maize, the former apparently consisting mainly of galactose units, the latter mainly of xylose. Since it seemed possible that the difference was between typical dicotyledon leaves and monocotyledon leaves (which may be flattened stems rather than true leaves), other monocotyledon leaves, hyacinth and narcissus, were examined; these however gave hemicelluloses resembling those of the bean.

Apart from the leaf products, hemicelluloses from other non-lignified tissues were also examined. Schulze and Pfenniger had already reported that the products isolated from bean and pea pods consisted of galacto-arabans; determination of carbon dioxide in the products showed the presence of considerable amounts of uronic anhydride, identified as that of galacturonic acid.

It was noted, finally, that the hemicelluloses of the radish root (or swollen hypocotyl) were markedly different from those of the leaves, in that fraction "A" contained proportions of pentosan (or uronic anhydride).

The presence of an insoluble hexosan in the insoluble hemicellulose fraction was unexpected, the previously recorded insoluble hemicelluloses having consisted mainly of xylans, with but small amounts of uronic anhydride and no hexosan. It may be suggested that insolubility is characteristic of true sugar anhydrides of high molecular weight (polysaccharides), whether hexosans or pentosans; such a grouping would include the starches and celluloses, as well as the xylans and the galactans here described. On the other hand, the soluble hemicelluloses may comprise those substances containing a sufficient proportion of uronic acid groups (*i.e.* the acid saccharides or polyuronides) and their solubility may be dependent on the presence of these (polar) carboxyl groups in the molecule. In this connection, it may be observed that the oxycelluloses, which undoubtedly contain glycuronic anhydride units [Kalb, 1927], are much less insoluble than true cellulose. If this conception holds, then the water-soluble ϵ-galactan of Schorger and Smith [1916] should be an acid saccharide, and the fact that Schorger found therein some 6 % of furfuraldehyde but was unable to isolate any pentose sugar may thus be explained. The insolubility of pectic acid, however, with its very high proportion of carboxyl groups, remains unexplained.

Table IX. *Constituents of leaf and other hemicelluloses.*

Tissue	Hemicellulose fraction	Sugars *etc.* present	Furfuraldehyde yield %	Uronic anhydride content %
Bean leaf	"A"	Galactose	0	0
,,	"B"-"C"	Galactose, arabinose, galacturonic acid	18·4	19·6
Vine leaf	"A"	Galactose, ? hexose*	0	0
,,	"B"-"C"	?	6·7	15·2
Radish leaf	"A"	Galactose	0	0
,,	"B"-"C"	?	21·0	—
Maize blade	"A"	Xylose, uronic acid	45·7†	<5†
,,	"B"-"C"	Xylose, arabinose, uronic acid hexose?		
Hyacinth	"A"	Galactose	0	0
Radish root	"A"	?	9·0	—
,,	"B"-"C"	?	15·0	—
Bean pods	Mixed	Galactose, arabinose, galacturonic acid	21·0	11·2
Sweet pea pods	Mixed	?	33·0	14·0
Rose shoot	Mixed	?	32·5	16·4

* See p. 207. † Average for mixed hemicelluloses as isolated.

Table X. *Pectic substances of leaves.*

Leaf	Type of leaf	Total pectin (as Ca pectate) % of dry leaf
Bean	Normal	6·5–8·9
Sycamore*	Normal	5·9
Vine	Acid leaf	6·9
Ivy*	Evergreen	7·2
Maize	Monocotyledon	1·0–1·7
Hyacinth	Monocotyledon	9·2
Narcissus	Monocotyledon	10·3
Cabbage	Inner (etiolated)	7·4

* *Cf.* figures given by Nanji and Norman [1928].

A summary of the observations on the constituents of these leaf and other hemicelluloses appears in Table IX, while in Table X are given some collected data, arrived at in the course of the present work, concerning the little known subject of the distribution of pectin in leaves.

DISCUSSION.

Dealing in the first place with the observations on pectins and hemicelluloses only, it is evident that the frequently-made statement, that the two classes are complementary, needs some modification, for in some tissues (*e.g.* bean pod) both groups are present in very large amount, the quantities of each being in fact very similar. In this tissue, development of the two classes has been shown to proceed throughout the whole of the period of growth, and it is only at the final stages of ripening that the proportion of the two becomes altered, some of the hemicellulose being lost. In non-lignified tissues in general it seems that pectins and hemicelluloses of the galacto-araban type may exist together in any proportions; for instance, comparing the figures given for pods of beans and sweet peas, the total combined content of the two groups is roughly 30–35 % in each tissue, but whereas the pectin/hemicellulose ratio in the bean is roughly 1/1, in the sweet pea it is approximately 1/9. In lignified tissue, on the other hand, pectin certainly is usually present in but small amounts, and this statement is true both for material of considerable age (timber) and for comparatively young growths (grass, maize), the hemicelluloses greatly predominating, according to the usual conception.

There seems however to be a further distinction between lignified and non-lignified tissues in the nature of their hemicelluloses. The analyses here recorded as well as others given in the literature show that lignified tissues are characterised by the presence of xylan and allied hexo-pentosans of the same series (glucosan—glycuronic anhydride—xylan) while in the non-lignified material galacto-arabans constitute the main bulk of the hemicelluloses. The information to be found in the literature is somewhat indefinite, but in the cases examined this generalisation is found to hold. The distinction is of course not rigid but is sufficiently evident. A brief summary of the available data is given in Table XI.

It would seem best therefore to look upon pectins as comprising a limited number of galacto-arabans of a rather special type, arising in certain circumstances during the course of the transformation of galactans into arabans. With regard to the possible conversion of pectins into bodies of the hemicellulose type, it is evident that from the former only substances of the araban type are likely to arise; this follows from the structural relationships of the various sugars entering into the composition of these substances, if the usually accepted view of the oxidation—decarboxylation mechanism already outlined (p. 197) be followed. Thus the typical major hemicellulose component of lignified tissues, xylan, would not be formed as the result of pectin decarboxylation. Suggestions that hemicelluloses in general may arise thus from pectin have therefore been based on the unfortunate lack of conciseness in the application of the term "hemicellulose," which has been adopted to include a widely differing collection of substances, with often only certain physical properties in common.

Moreover, from the results of the experiments here recorded, it would seem that the conversion of pectin *in vivo* into other substances of whatever nature is by no means so easy as has been suggested. The persistence of the pectins during the period of development of tissues (bean pods, shoot of rose *etc.*) and under the artificial conditions of starvation and of slow drying points to a marked degree

Table XI. *Collected data concerning distribution of galacto-arabans and xylans in various tissues.*

Material	Authority	Galacto-arabans, including pectins, approx. % of tissue	Xylans, approx. %	Type of tissue
Bean leaf	(This work)	*ca.* 12	?	Non-lig.
Bean pod	(This work)	*ca.* 30	?	Non-lig.
Sweet pea	(This work)	*ca.* 30	?	?
Iceland Moss	Buston and Chambers [1933]	*ca.* 10	0	Non-lig.
Various fruits	—	15–35	—	Non-lig.
Grass	Buston [1934]	*ca.* 6	*ca.* 20	Lig.
Oat-straw	Norman [1929]	(Pectin, 1 %; galactose, arabinose and xylose all present; amounts not stated)		Lig.
Maize	(This work)	(Pectin 1 %)	*ca.* 20	Lig.
Maize-cobs	Preece [1930]	(Mainly xylan; some araban)		Lig.*
Wheat-bran	Norris and Preece [1930]	(Mainly xylan; some araban)		?
Apple wood	Tottingham *et al.* [1920]	(Mainly xylan; some galactan)		Lig.
Oak	O'Dwyer [1923]	*ca.* 3	*ca.* 15	Lig.
Beech	O'Dwyer [1926]	*ca.* 2	*ca.* 5	Lig.
		(amounts isolated on large scale)		
Other woods	—	(Mainly xylan)		Lig.

* 15 % lignin [Schorger, 1926].

of stability; pectin transformation, where it occurs, must therefore normally be a process taking place during a prolonged period. Some interesting figures in this connection are quoted by Schorger [1926, p. 113], which, although the assumptions upon which they are based are open to question, tend to indicate again the comparatively great stability of the pectins.

As the pectic substances in the tissues are considered to be present within the cell-walls, partly as middle lamella substance and partly in definite combination with cellulose, they are to a large extent not readily exposed to the oxidising mechanism of the cell. Alternatively, the stability of the pectins may be due to the fact that, as Sucharipa [1924] has shown, in the case of *Citrus* fruits, the pectins in the cell-wall are esterified, through their carboxyl groups, with cellulose residues, and this association may render the carboxyl groups resistant to attack, although as Norman and Norris [1930] have shown free pectin molecules are extremely easily oxidised *in vitro*.

In contrast to the apparent stability of the pectins, some at least of the hemicelluloses seem open to chemical action, shown partly in intramolecular changes of the substances themselves (as evidenced by the alteration of their apparent pentosan content *etc.*) and partly in their disappearance from the tissues under certain conditions. This disappearance may be due to their mobilisation for purposes of oxidation (respiration) as in the bean leaves during starvation; or to their translocation to another part of the plant, as suggested by Schulze and Pfenniger in the case of bean and pea pods and again indicated in the present work. The changes in structure of the hemicelluloses have been shown to affect mainly the hexosan part of the molecule, although in some cases there also appeared to be a net loss of pentosan (Table V*a*). The fact that the hemicelluloses of maize, in contrast to those of bean and vine leaves, remained but little affected by the starvation process was perhaps to be expected in view of their much smaller content of hexosan; their chief component, xylan, being unaffected by the experimental conditions.

Returning to the consideration of the relation between pectins and the other galacto-arabans found in the plant tissues, it would be expected that, if all such compounds are the derivatives of a parent galactan (or series of galactans of different molecular weights), the pectins would be formed under conditions conducive to extreme oxidation, without subsequent decarboxylation. That these conditions may be produced during rapid growth in the presence of an abundant water supply (as already suggested by various authors) would seem to be indicated by the known relative distribution of the pectins and of the other galacto-arabans. Examples may be seen, in the present work, in the results of the experiments on radishes, and also in comparing the distribution of the types of compound in the pods of beans and sweet peas respectively—the former having a much more rapid growth rate, together with a greater water supply, than the latter.

More detailed suggestions as to the exact course and nature of the oxidation-decarboxylation process are not possible. Norman and Norris suggest that the latter process is actually a prolonged mild oxidation, but it is difficult to see, if such were the case, why decarboxylation should occur when but a small proportion of galactan units, in some types of molecule, have undergone the primary oxidation; one would rather expect this first type of oxidation to have reached its limit before the presumably slower type of oxidation, leading to decarboxylation, became evident.

With more knowledge of the chemistry of the hemicelluloses and polyuronides generally, it would seem that the time is approaching when the designation of hemicellulose fractions as "A," "B" *etc.* on a solubility basis should be modified. Members of the insoluble ("A") type hitherto described have proved to be mainly pentosans, with at most a low uronic anhydride content. From leaves, another type of substance, which on grounds of solubility would be classed as "hemicellulose A," has now been isolated; chemically, however, it is quite unlike the various hemicelluloses "A" previously recorded. A more satisfactory basis of classification, if it could be applied, would recognise the following types: (I) compounds predominantly hexosan; (II) intermediate compounds comprising hexosan-uronides, pentosan-uronides *etc.*; these might be of any degree of oxidation, from the but slightly oxidised compounds of the type isolated from lichens by Buston and Chambers [1933] to the more complex bodies in which a considerable amount of pentosan formation has occurred; and (III) compounds predominantly pentosan, such as the xylans of maize-cobs, straws *etc.* Further, examples of each of the three types could arise from glucose, galactose or other parent hexoses. Thus pectin would become a particular member of type II of the galactose series. Pectin itself seems to be widely regarded as an entity, the definite formula of Nanji *et al.* [1925] having found acceptance, although there is evidence that pectins from various sources may differ somewhat in their structure. It is agreed however that they are members of the galactose-uronide series and that their characteristic is a high state of oxidation, as evidenced by their high content of galacturonic anhydride; and perhaps it is best to regard them as comprising a sharply defined group of highly oxidised galacto-arabans.

In order to pass from a galactan (type I) to an araban (type III) it would not be necessary to pass through pectin as an intermediate—indeed, direct observations on these compounds *in vivo* suggests that passage into pentosans is actually more easy *via* intermediate compounds of a lower state of oxidation than *via* pectin. Thus the araban isolated by Ehrlich [1930] from beet-pulp is not necessarily derived from the pectin with which it is associated (see also Colin and Chaudin [1934]).

It would seem that the ability to form stable compounds with a very high uronic anhydride content such as the pectins is confined to the galactose series, for no glucose or mannose polyuronides have been isolated having a similar constitution. In the xylan series, in fact, the process seems to tend to pass rapidly through to the pentosan stage, glycuronic anhydrides being of comparatively rare occurrence among plant products and the glucosan-hemicelluloses still more so. The parent hexosan of the xylans is possibly cellulose itself, but this seems hardly likely, for xylan is frequently found in very close association with cellulose and is in fact almost certainly derived from it *via* glycuronic anhydride units; this xylan differs from the xylans isolated as "hemicelluloses" in its extreme resistance to extraction.

Turning to the question of lignin development, in view of the relative lack of knowledge of lignin itself, speculation must necessarily be more tentative. As has been pointed out, the evidence in favour of the theory that lignin arises by a transformation of pectin cannot be ignored. The microchemical evidence put forward by Ritter [1925], that pectin is absent from woody tissues in general and especially from the middle lamella, does not however prove that it is replaced by lignin. The actual isolation of pectins from wood does not altogether accord with the conclusions of Ritter.

It is admitted that, although pectin is remarkably stable in the tissues, over comparatively short periods, there is no reason to suppose that it is completely exempt from the general process of decarboxylation. Its final state is probably to be sought in the small amounts of arabans which are to be found in all woods, and which microchemical tests are not adequate to identify.

The observed possession by pectin and by lignin in common of hydroxyl and methoxyl groups is still less evidence of the supposed transformation, even though the latter constitute similar proportions of the molecule in the two cases. For the methoxyl of pectin is readily removed, *e.g.* by enzyme action, and yet it is supposed to persist in the molecule throughout the admittedly profound changes involved in lignification. In any event, it seems that the first step in pectin transformation consists of a demethylation (as indicated by the relative proportions of pectate-pectin in grass and hay respectively and by the methoxyl contents of the grass and hay pectins as isolated). Further, in his experiments on the extraction of pectin from lemon albedo, Sucharipa [1924] found that only a small amount of pectin was present in the fully esterified form (11·7 % OMe) and that only in the most easily extracted portion. The main bulk of the pectin was present in a form in which much of the methoxyl was apparently replaced by cellulose residues. It seems more likely that the methyl groups are split off from the pectins during the period of transformation and subsequently become combined with the unknown lignin precursor.

With regard to the arguments of Ehrlich [1930] based upon his observations on flax pectins, the reputed presence of xylose in the pectin molecule seems remarkable. No other example of a xylose-containing pectin has been reported elsewhere, so far as can be ascertained. It is true that flax pectin has been considered to be of abnormal type [Henderson, 1928] but the abnormality reported has suggested a simpler structure than usual, and not one more complex as would be required by the inclusion of xylose in the molecule. The question of the constitution of flax pectin has been examined also by Norris [1929] who failed to detect any abnormality. It would seem more likely that the xylan in Ehrlich's product is an accompanying impurity, extracted simultaneously from the tissues —xylans, as already pointed out, being typical of lignified material such as flax stems. The methods of separation of pectins, excluding that of conversion into calcium pectate, which is not favoured by Ehrlich, are mainly of physical type (based on solubilities *etc.*), and some of the xylans might well have solubilities similar to that of pectin. The lignin-like body separated by Ehrlich may also be but physically associated with pectin, or, if actually in chemical combination, not necessarily derived from pectin. Ehrlich emphasises the facts of its solubility when present in association with the pectin and its loss of solubility on separation and sees in these evidence for its derivation from pectin. The soluble form of cellulose described by Bailey and Norris [1932] and others, which is present in seed mucilages, shows a similar phenomenon in losing its solubility on isolation. There is however no suggestion that it is derived from the other constituents of the mucilage—indeed since the latter consist mainly of pentosans, such an origin is evidently out of the question. Nor has the fact that pectin appears chemically combined with cellulose in protopectin [Sucharipa, 1924] been used as evidence that pectin is a cellulose derivative. Had Ehrlich's product been a link between pectin and lignin, it would probably have been met with elsewhere, in view of the large number of examinations of woods at all stages of growth that have been made.

The experiments recorded in the present paper do not suggest that pectin is the precursor of lignin in the tissues examined. In the case of the rose, considerable formation of lignin occurred with no corresponding loss of pectin, the amount of which indeed increased, though at a slower rate. The main change observed in the rose shoot consisted in a fairly rapid growth, not in the sense of an elongation of the shoot, but in the sense of formation of dry matter, that is, by laying down of fresh material on to the cell-wall structure already present. Of the materials so laid down, lignin accounted for 1·6 g., while the pectin content rose by 0·3 g. It may be argued that the pectin was in a state of continual formation and transformation, while maintaining a fairly constant balance. This would involve a marked lability on the part of the pectin molecule, such as does not appear from the experiments on detached leaves.

It may be pointed out that lignification must be a more rapid process, at least in its first stages, than is sometimes realised; for Mehta [1925] has shown the presence of lignin in quite young pine needles, and there is evidence of its presence in grasses at an early stage. On the other hand, pectin breakdown or alteration, though not excluded, is slow.

While therefore the evidence is not satisfactory in support of the view that pectin is the precursor of lignin, it is evident that the latter must be derived finally from some sugar, and probably through a polysaccharide. The experiments of Hawley and Wiertelak [1931], although possibly of a rather artificial type, suggested that the source of the lignin was to be found in certain pentosans. The amount of pectin and galacto-arabans present in their original ashwood was almost certainly insufficient to account for the amount of "lignin" they obtained.

In view of the predominance of xylans in lignified tissues, these, or closely related bodies, seem more likely to be the precursors of lignin. It has been pointed out that xylan formation seems to take place more rapidly than the corresponding change to araban; this suggests a degree of lability in the precursors of xylan which may bear upon the comparatively rapid rate (in some cases, at least) of lignification. The view that xylan is the main precursor of lignin has been advanced by Klason [1930]. However, as no attempt has been made in the present work to examine these suggestions, it is not appropriate to discuss them here.

Summarising the views here expressed, the following suggestions may be put forward.

(i) If glucose be regarded as the primary sugar formed in the plant, part of this becomes converted into galactose, the two sugars thus becoming severally the precursors of the glucosan—xylan and galactan—araban series of polysaccharides. Since the latter are more characteristic of non-lignified tissues, it would seem that there is some connection between formation of galactose or galactans and development of a non-lignified structure. Nevertheless, increasing age, or an alteration in the conditions of the tissue, may result in decreasing galactose formation, together with increasing predominance of xylan production.

(ii) Of the galactan series, the pectins arise under rather exceptional conditions, involving rapid growth, combined with a copious water supply; while the other galacto-arabans, of a lower degree of oxidation, are formed when metabolism is at a different, lower level. Finally, all these bodies may pass into true arabans, but the pectins, either by reason of some innate stability of the molecule (alone or in combination with cellulose), or by reason of their location in the cell-wall, are only slowly transformed. In this limited sense, pectins are the precursors of hemicelluloses, but only of a small range of these substances, *viz.* the arabans.

(iii) The glucosan-xylan series is not apparently typical of rapidly growing tissues, or of tissues with a high water content. The intermediate members of the series are to be found in a less highly oxidised state than the corresponding galactans and much more readily pass into the final products of decarboxylation, the xylans.

(iv) Lignin does not arise from pectin, but more probably from some carbohydrate related to the glucosan-xylan series, with members of which series it is usually associated in the plant.

SUMMARY.

1. The distribution of pectin and of the hemicelluloses in developing pods of beans and sweet peas has been investigated. The proportion of each remains almost constant throughout the greater part of the growth period, but there is a falling off in the content of the latter towards the close of the period. This is possibly due to a translocation of some of the sugar constituents to the seeds.

2. Similar experiments have been made on the distribution of these and other cell-wall constituents during the development of a woody stem (rose). Here, the increase in lignin was found to run parallel with an increase in hemicellulose, but there was no loss of pectin. It is considered that pectin does not constitute the source of lignin during the primary lignification.

3. Detached leaves, submitted to a process of starvation, or of slow drying, showed no appreciable change in their pectin content, but the amount of hemicellulose showed a significant variation; hemicelluloses are therefore considered to be more labile than the pectins. It is possible that they may be in part available for respiration under certain circumstances. The main change during starvation was shown to consist in loss of hexose units, together with some decrease in pentosan.

4. Rapid growth of the plant is conducive to the formation of galacto-arabans, but especially of pectins.

5. The nature of the hemicelluloses of certain leaves has been examined. The "A" type has been shown to differ from those of lignified tissues in consisting almost entirely of hexosan units based on galactose. A modified classification of these bodies is tentatively suggested.

6. The bearing of these experiments on the possible transformation of pectins into hemicelluloses and lignin in the plant, and on certain theories of lignification, is discussed.

The author wishes to record his great gratitude to Dr V. H. Chambers and to Mr H. Thring, for their assistance in the experiments described in sections 4 and 6; and to Mr W. Hales and his staff at the Chelsea Physic Garden for providing much of the material used in the work.

REFERENCES.

Ahrns (1924). *Bot. Arch.* **5**, 234.
Bailey and Norris (1932). *Biochem. J.* **26**, 1609.
Browne (1912). Handbook of sugar analysis. (New York.)
Buston (1934). *Biochem. J.* **28**, 1028.
—— and Chambers (1933). *Biochem. J.* **27**, 1691.
Candlin and Schryver (1928). *Proc. Roy. Soc. Lond.* B **103**, 365.
Chibnall (1924). *Biochem. J.* **18**, 395.
Colin and Chaudin (1934). *Compt. Rend. Acad. Sci.* **198**, 2116.
Deleano (1912). *Jahr. wiss. Bot.* **51**, 541.
Dorée (1933). Methods of cellulose chemistry. (London.)
Ehrlich (1930). *Cellulosechem.* **11**, 161.
—— and Schubert (1926). *Biochem. Z.* **169**, 13.
—— —— (1928). *Biochem. Z.* **203**, 343.
Emmett and Carré (1926). *Biochem. J.* **20**, 564.
von Fellenberg (1918). *Biochem. Z.* **85**, 45, 118.
Fleischmann (1912). *J. Amer. Chem. Soc., Abstr.* **6**, 1919.
Fuchs (1927). *Biochem. Z.* **180**, 30.
van der Haar (1920). Anleitung zum Nachweis, zur Trennung und Bestimmung der reinen und aus Glukosiden u.s.w. erhaltenen Monosaccharide und Aldehydsaüren. (Berlin.)
Hawley and Norman (1932). *Cellulosechem.* **13**, 88.
—— and Wiertelak (1931). *J. Ind. Eng. Chem.* **23**, 184.
Henderson (1928). *J. Chem. Soc.* 2117.
Kalb (1927). *Ber. deutsch. chem. Ges.* **60**, 2514.
Klason (1930). *Ber. deutsch. chem. Ges.* **62** B, 1548.
Linggood (1930). *Biochem. J.* **24**, 262.
McDougal, Richards and Spoehr (1919). *Bot. Gaz.* **67**, 405.
Mehta (1925). *Biochem. J.* **19**, 979.
Nanji and Norman (1928). *Biochem. J.* **22**, 596.
—— Paton and Ling (1925). *J. Soc. Chem. Ind.* **44**, 253 T.
Norman (1929). *Biochem. J.* **23**, 1313.
—— and Norris (1930). *Biochem. J.* **24**, 402.
Norris (1929). *Biochem. J.* **23**, 195.
—— and Preece (1930). *Biochem. J.* **24**, 60.
O'Dwyer (1923). *Biochem. J.* **17**, 501.
—— (1925). *Biochem. J.* **19**, 694.
—— (1926). *Biochem. J.* **20**, 656.
—— (1928). *Biochem. J.* **22**, 381.
—— (1932). *J. Soc. Chem. Ind.* **51**, 968.
Preece (1930). *Biochem. J.* **24**, 1304.
Ritter (1925). *J. Ind. Eng. Chem.* **17**, 1194.
Schorger (1926). Chemistry of cellulose and wood. (New York.)
—— and Smith (1916). *J. Ind. Eng. Chem.* **8**, 494.
Schulze and Pfenniger (1910). *Z. physiol. Chem.* **68**, 93.
Spoehr and McDougal (1918). *Plant World*, **21**, 245.
Sucharipa (1924). *J. Amer. Chem. Soc.* **46**, 145.
Tottingham, Roberts and Leptovsky (1920). *J. Biol. Chem.* **45**, 407.
Widdowson (1931). *Ann. Bot.* **183**, 597.

XXVI. STUDIES ON THE ORIGIN OF SULPHUR IN WOOL.

II. A CUPROUS MERCAPTIDE METHOD FOR THE DETERMINATION OF CYSTINE OR CYSTEINE.

By SAREL DANIEL ROSSOUW
AND TOBIAS JOHANNES WILKEN-JORDEN.

From the Department of Agriculture, Veterinary Research Institution, Onderstepoort, South Africa.

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Having established the value of the Sullivan method, as modified by the authors [1934], as a specific and quantitative reaction for the determination of cysteine or cystine, it became necessary to study its possible application to the analysis of animal and vegetable materials. The first step in such an analytical procedure would obviously include some process such as acid hydrolysis whereby the cystine or cysteine of the raw material is totally liberated and quantitatively brought into solution. In such processes the intensity of acid treatment must be sufficient to liberate all the cystine or cysteine from its protein or protein-like complex, but not so intense as to destroy the cystine thus liberated. It is not the purpose of the present paper to consider this phase of the problem, since investigations in this direction are still under way. On the contrary, it has been considered advisable to assume, for the time being, that the hydrolysis of the raw material can be effected quantitatively. What has been aimed at in the present work is to find a method whereby the cystine or cysteine actually present in such hydrolysates may be determined successfully.

In a few cases of high cystine content, *e.g.* wool and some pure proteins rich in cystine, the colour of the hydrolysate causes very little interference, and the modified Sullivan technique can be applied directly and with satisfactory results. With low cystine content and a dark-coloured hydrolysate, the problem becomes rather involved. It is obviously quite impossible to apply directly a colour reaction to a hydrolysate obtained from grass or some animal tissue low in cystine, as anyone who has handled such solutions will readily appreciate. To overcome this difficulty it has been suggested by Henrici [1932] that the cystine should first be precipitated from such a solution. Even after much manipulation it is still difficult however to obtain a solution sufficiently light in colour for colorimetric methods to be applied with any degree of satisfaction. Certain workers [Sullivan, 1929; Sullivan and Hess, 1930] have suggested the use of active and animal charcoal and other decolorising agents. None of these substances however appears suitable, since the cystine in solution is either adsorbed along with the colouring matter, or it is catalytically decomposed and destroyed [Baur and Wunderly, 1933]. Other workers [Lugg, 1933] have tried to inhibit colour development during hydrolysis by adding such reducing agents as stannous or titanous chloride with equally unsuccessful results.

On the other hand it has been shown by Vickery and White [1932], amongst others, that cysteine, in quantities of about 20 mg., can be precipitated quantitatively from solution as the insoluble cysteine cuprous mercaptide. Vickery and White used cuprous oxide, and determined the cystine in the solution from the sulphur content of the cuprous mercaptide precipitate. Hopkins [1929] precipitated glutathione in a similar way and found the compound to be $C_{10}H_{16}O_6N_3SCu$. Preliminary experiments on the behaviour of this cuprous mercaptide soon led to the conviction that it embodied a reaction sufficiently sensitive to enable the separation of quantities of cystine or cysteine much smaller than that postulated by Vickery and White.

The addition of cuprous oxide in the solid form appeared somewhat cumbersome and unsuited for micro-work. Since cuprous chloride is only very slightly soluble in water, initial attempts were made with cuprous chloride dissolved in potassium acetate and acetic acid mixtures. Although promising results were sometimes obtained with quantities as low as 1 mg. of cystine reduced to cysteine by means of a few mg. of zinc dust, the results were somewhat irregular. At this stage it was resolved to study the precipitation of cystine by making use of the well-known solubility of cuprous chloride in potassium chloride solutions. A very interesting fact was soon discovered. Working on 2 mg. quantities of cystine per aliquot volume, the preliminary reduction to cysteine was omitted by mere accident; however, on adding the solution of cuprous chloride in potassium chloride, the cysteine cuprous mercaptide was observed to form as readily as if all the cystine had been present as cysteine. In all subsequent experiments the preliminary reduction of cystine to cysteine was therefore omitted. The cuprous chloride solution was prepared from Merck's Reagent cuprous chloride. About 0·5 g. of the salt was shaken up with 1 % HCl to remove most of the cupric chloride present, the insoluble cuprous chloride allowed to settle, and the supernatant liquid poured off. This perfectly white residue was then dissolved in a minimum quantity of 25 % KCl containing 0·2 % HCl. The reagent should, preferably be prepared freshly before use, although, if left in a well-stoppered bottle it may be kept for a few days.

On studying the precipitation of cystine with this reagent, it was found that the cysteine could be quantitatively precipitated at room temperature at hydrogen ion concentrations between p_H 3·0 and 5·5, although the rate of precipitation appeared to be greatest at p_H 4·5. It was therefore decided to work at a hydrogen ion concentration of approximately p_H 4·5. Aliquot portions of 2 mg. cystine dissolved in very dilute HCl were measured into 50 ml. centrifuge-tubes and a few drops of glacial acetic acid added to each. These solutions were then brought to p_H 4·5 by adding carefully 10 % KOH using bromocresol green (B.D.H.) as an external indicator. To the buffered solution cooled to room temperature 5–10 drops of the cuprous chloride solution were added with constant stirring. A fine precipitate was observed to form almost immediately and coagulated gradually to a flocculent mass. After 40 minutes this precipitate was centrifuged and the supernatant liquid poured off. The precipitate was washed with alcohol, again centrifuged and the alcoholic liquid poured off. After draining, the precipitate was dissolved in 5 ml. 1–2 % HCl, and transferred to a 25 ml. measuring cylinder of good accuracy, using about 15 ml. of wash water. 2·5 ml. of a 5 % acetic acid solution were then added, and after mixing 1 ml. of 10 % KCNS was added in order to precipitate the cuprous copper. To remove the varying amounts of cupric copper always present at this stage, use was made of the reaction of Biazzo [1926] by precipitating the cupric copper as the green sulphocyanide-copper-pyridine complex. At this stage pyridine was therefore added until p_H 4·5 was reached. Under these conditions no measurable adsorption of cystine by the precipitate could be detected. After mixing thoroughly and making up to 25 ml., the precipitates were filtered off. To overcome the buffering action of the acetic acid in solution, 1 ml. of 10 % KOH was added to a suitable aliquot (usually 5 ml.) of the clear filtrate, and the cystine was determined by the modified Sullivan method [Rossouw and Wilken-Jorden, 1934].

Using this method, the effect of varying amounts of potassium chloride on the precipitation of the cysteine cuprous mercaptide was studied, since with actual hydrochloric acid hydrolysates the amount of potassium chloride in the aliquot solution must vary with the amount of cystine present in the material. This consideration is most important, since, as the curves reproduced below readily show, the cuprous mercaptide will partly or totally dissolve in an excess of potassium chloride, unless a correspondingly large excess of cuprous chloride reagent is added. However, considering the fact that the presence of potassium acetate in relatively large quantities has been found to depress the solubility of the mercaptide in potassium chloride, the curves show that it is perfectly practicable to determine the cystine in such solutions as a hydrochloric acid hydrolysate of sheep's blood. In dealing with grasses however the position is somewhat different, since in a hydrochloric acid hydrolysate a suitable aliquot containing a determinable amount of cystine would inevitably contain such a large quantity of hydrochloric acid that the amount of cuprous chloride reagent necessary would become unwieldy and impracticable.

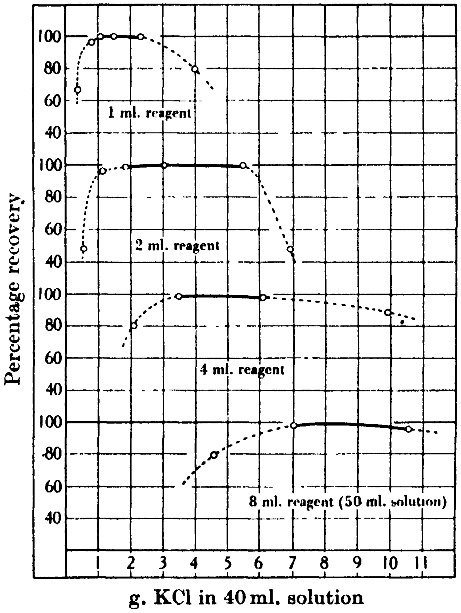


Fig. 1.

To overcome this difficulty when working with material very low in cystine, the hydrochloric acid was replaced by sulphuric acid. In testing this modification of the method 2 mg. of cystine were added to 5 ml. of 40 % H_2SO_4, 2·5 ml. glacial acetic acid were added, and the solution was brought to p_H 4·5 by neutralising with 10 % KOH. After cooling to room temperature, 5–10 drops of the cuprous chloride solution were stirred in. Precipitation and flocculation were rapid, and

100 % recovery was obtained repeatedly, even after leaving the precipitate to stand for 24 hours.

This procedure of first precipitating the cystine as the cysteine cuprous mercaptide soon proved most successful in the treatment of hydrolysates, especially those highly contaminated with excessive quantities of dark colouring and other matter. On bringing such hydrolysates to p_H 4·5 by means of acetic acid and potassium hydroxide, a large amount of objectionable colouring material was precipitated and centrifuged off. That the material thus precipitated could occlude or contain no measurable amount of cystine can be safely accepted, since neither could cystine be found in such precipitates nor could any loss be demonstrated on adding a known amount of cystine to the hydrolysate. On adding the cuprous chloride reagent to the centrifuged and decanted solution, a further quantity of dark-coloured material was precipitated along with the cuprous mercaptide. On dissolving the mercaptide in dilute hydrochloric acid, the accompanying dark material was also brought into solution, but was removed practically completely in the precipitation of the copper with sulphocyanide and pyridine. The final solutions were either perfectly colourless or exhibited only the faintest straw-yellow which has no measurable influence on the cystine determination. As an example of the applicability of the method, the following analytical figures may be given:

Merino wool	12·5 %
Dried sheep's blood	450 mg./100 g.
Yellow mealies	104 „
Lucerne hay	80 „
Veld grass...	20–80 mg./100 g.

In the successful execution of the method, some experience is undoubtedly necessary. If too large an excess of the cuprous chloride reagent be added to the sulphuric acid hydrolysates, some cuprous chloride may be precipitated along with the mercaptide. Excessive quantities of this cuprous chloride make the dissolution of the mercaptide in the dilute hydrochloric acid more difficult to control and may further result in some cystine being retained by the cuprous chloride. In such cases the difficulty may be remedied by adding a few mg. of zinc dust to the hydrochloric acid solution and thus converting the cuprous chloride into metallic copper. In such cases however the filtrate, after adding sulphocyanide and pyridine, must be left to stand overnight to allow any cysteine formed to be reoxidised to cystine. Any zinc in solution is practically completely removed by pyridine at p_H 4·5; the residual traces that may remain in solution have no effect on the determination by the Sullivan method, as also shown by Prunty [1933].

Method.

The method, employing sulphuric acid, may be summarised as follows.

A suitable quantity of dried material *plus* 8 times its weight of 40 % H_2SO_4 hydrolysed for 24 hours at 125° (oil-bath temperature).

Hydrolysate filtered hot and made up to volume.

A suitable aliquot measured into a 50 ml. centrifuge-tube. 2·5 ml. glacial acetic acid added to this and brought to p_H 4·5 with 10 % KOH.

If precipitate is shown here, centrifuge and wash with acetic acid-KOH mixture of p_H 4·5 (final volume to be approximately 40 ml.).

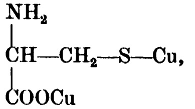
Stir in a few drops of cuprous chloride reagent.

Leave until flocculation sets in (a few minutes to 1 hour).

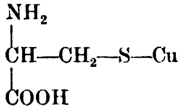
Centrifuge and wash with alcohol.
Dissolve in 5 ml. 1 % HCl.
Transfer, with washings, to a 25 ml. measuring cylinder.
Add 2·5 ml. 5 % acetic acid, and mix.
Add 1 ml. 10 % KCNS and mix.
Add pyridine to p_H 4·5 and make up to volume.
Filter.
Determine cystine in suitable aliquot (0·4 mg. cystine), by method of Sullivan as modified by the authors, adding 1 ml. of 10 % KOH to counteract buffering effect of acetic acid.

Discussion of mechanism.

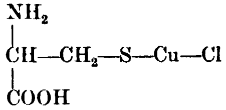
The exact nature of the cuprous mercaptide precipitate has not been determined. Indications [Preisler and Preisler, 1932] have been obtained that the mercaptide which is either



$\underset{\displaystyle COOCu}{\overset{\displaystyle NH_2}{CH}}-CH_2-S-Cu,$

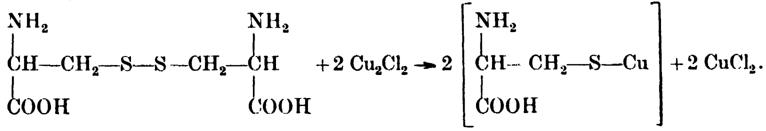


$\underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}}-CH_2-S-Cu$ or



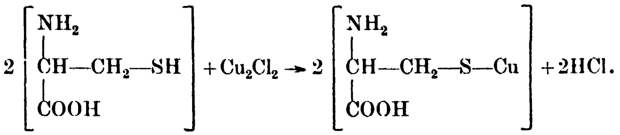
$\underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}}-CH_2-S-Cu-Cl$

is apparently associated with either cuprous or cupric chloride, or both. Whatever the composition of the precipitate, it is generally accepted that all the cystine is present in the reduced form as the copper salt of the mercaptan cysteine. It therefore appears that the cystine reacts with the cuprous chloride according to the following reaction:



$$(1)\quad \underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}}-CH_2-S-S-CH_2-\underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}} + 2\,Cu_2Cl_2 \rightarrow 2\left[\underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}}-CH_2-S-Cu\right] + 2\,CuCl_2.$$

In the case of the preformed cysteine, the reaction would naturally be:



$$(2)\quad 2\left[\underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}}-CH_2-SH\right] + Cu_2Cl_2 \rightarrow 2\left[\underset{\displaystyle COOH}{\overset{\displaystyle NH_2}{CH}}-CH_2-S-Cu\right] + 2HCl.$$

These reactions seem to proceed quantitatively in feebly acid solutions (p_H 3·0–5·5) in the presence of an excess of cuprous chloride. In dissolving the precipitates in dilute hydrochloric acid, the initial state of the cystine should be the reduced form. Experimental data can be adduced however to show that its conversion into the oxidised form is extremely rapid in such acid solutions. In the normal course of analysis all the cysteine is quantitatively converted into cystine by the time the copper is precipitated with sulphocyanide and pyridine, even when operations are speeded up to the maximum limit with the object of detecting any unoxidised cysteine. It appears that the transformation to cystine already occurs during the process of dissolution in hydrochloric acid, since, even after adding small quantities of zinc powder no cysteine could be detected. This behaviour of the mercaptide suggests that it is precipitated as a complex containing cupric chloride. In the presence of hydrochloric acid, and in the absence of excess cuprous chloride, the first reaction is therefore reversed, the reaction being forced to completeness by the precipitation of the cuprous copper as sulphocyanide. The observation of Andrews [1933] on the catalytic oxidation of cysteine by copper must probably be explained in a similar way.

In the case where a relatively large excess of zinc dust is added to the solution of the mercaptide in dilute hydrochloric acid, it was found possible, by rapid work, to obtain practically all the cystine in the reduced form. Since in the Sullivan method the treatment with cyanide results in the formation of only one molecule of cysteine from one molecule of cystine, and since, for all practical purposes one part by weight of cystine is theoretically equivalent to one part by weight of cysteine, the Sullivan method in reality determines only half of the cystine when present as cystine. When present as cysteine therefore, a colour intensity double that of the standard should be obtained. In actual experiment colour intensities round about 192 were obtained with the standard at 100. On allowing the solution from which the copper had been precipitated to stand for a further 4 hours, the intensity of the Sullivan colour dropped to 150, while after 20 hours it had dropped further to 100, showing that by that time all the cysteine had been converted into cystine. Since some extractions with chloroform according to Biazzo still revealed minute quantities of copper in the solution, the conversion seems to have been effected catalytically.

The rapid conversion of the cysteine in the mercaptide into cystine was further demonstrated by precipitating the cysteine from a standard cysteine hydrochloride solution with cuprous chloride, and comparing the colour intensity of the cysteine treated in this way with that of the cysteine hydrochloride standard directly treated by the Sullivan method. The cysteine from the mercaptide was found to be only half that of the cysteine treated directly.

The fact however that in the hydrochloric acid solution of the mercaptide the cystine can be quantitatively reduced by means of zinc dust and the modified Sullivan method applied to this cysteine solution, is considered to be of some practical importance, since double the normal colour intensity is obtained. Along these lines it has been found possible to determine the cystine contents of grasses and other material extremely low in cystine. These and other results will be reported in a following paper.

SUMMARY.

1. A method for the micro-determination of cystine in biological or plant material has been described. The dried material is hydrolysed with 40 % sulphuric acid and the cystine precipitated by cuprous chloride as the cysteine cuprous mercaptide. The cystine content of this is determined by the modified Sullivan method.

2. The interfering colouring matter is automatically eliminated.

3. The method is quick and comparatively easy.

REFERENCES.

Andrews (1933). *J. Biol. Chem.* **100**, iv.
Baur and Wunderly (1933). *Biochem. Z.* **262**, 300.
Biazzo (1926). *Ann. Chim. Appl.* **16**, 2. Cited from Elvehjem and Lindow, *J. Biol. Chem.* **81**, 437.
Henrici (1932). *18th Rep. Dir. Vet. Ser. An. Ind. U. of S.A.* 579.
Hopkins (1929). *J. Biol. Chem.* **84**, 269.
Lugg (1933). *Biochem. J.* **27**, 1022.
Preisler and Preisler (1932). *J. Biol. Chem.* **95**, 181.
Prunty (1933). *Biochem. J.* **27**, 387.
Rossouw and Wilken-Jorden (1934). *Onderstepoort J. Vet. Sci. An. Ind.* **2**, 361.
Sullivan (1929). *Suppl. No. 80 to U.S. Publ. Health Rep.*
—— and Hess (1930). *Suppl. No. 86 to U.S. Publ. Health Rep.*
Vickery and White (1932). *J. Biol. Chem.* **99**, 701.

XXVII. THE MECHANISM OF THE INTERCONVERSION OF THE HEXOSE SUGARS IN LIVING ORGANISMS.

By KAMEL MIKHAIL DAÓUD and WADEI TADROS.

From the Biochemical Laboratory, Physiology Department, Faculty of Medicine, Cairo, Egypt.

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The transformation of one hexose sugar into another is a process which takes place in both kingdoms of living matter. Thus in animals, fructose, mannose and galactose when slowly introduced into the circulation form glycogen which is supposed to be a result of an intermediate formation of glucose. When lactose is administered to diabetics two molecules of glucose for each molecule of lactose may be recovered in the urine. This indicates that the tissues can under certain conditions transform galactose into glucose. The reverse process, that is the transformation of glucose into galactose, has been observed by Rohmann [1919], who shows that the galactose constituent of milk sugar is formed from *d*-glucose.

In plants, Daoud [1932], working on the germination of fenugreek seeds, found that while the reserve polysaccharide of these seeds is composed of mannose and galactose, the radicles of the germinated seeds contain glucose, fructose and sucrose, this last sugar being entirely absent from the ungerminated seeds. The reverse process undoubtedly takes place during the development of the seed, *i.e.* the galactose and the mannose constituents of the reserve polysaccharide must arise from the photosynthetic sugars.

The mechanism of the transformation of one hexose into another is the subject of the present investigation. The transformation of a sugar such as mannose into its epimeride (glucose) may to some extent be explained by the ease with which the reversal of the stereochemical configuration of carbon atom 2 takes place. This occurs *in vitro* under the action of alkalis on either of these two sugars; but as a result of this reaction a mixture of these three and fructose together with other sugars is formed. The fact that the mannose constituent of the reserve polysaccharide of fenugreek seeds is not found in the free state among the other hexoses during germination suggests that the mechanism *in vivo* is different from that *in vitro*. Still more difficult to understand is the transformation of a sugar like galactose into glucose or *vice versa*.

Robinson [1927, 1], bearing in mind the common occurrence of hexosephosphates in nature, suggests that the transformation of glucose into galactose takes place through the reversal of the stereochemical configuration of carbon atom 4 on the hydrolysis of glucose-4-phosphate; the reverse change will have the same mechanism.

The view of Robinson was opposed by Levene [1927] who observed that glucose-3-phosphoric acid ferments at the same rate as glucose. Should glucose-3-phosphoric acid on hydrolysis pass into allose, then the rate of fermentation would be lower than that of glucose. Furthermore, Raymond and Levene found that on hydrolysis of glucose-3-phosphoric acid, only one sugar, namely glucose, is obtained.

Robinson [1927, 2] brings into consideration firstly, that even if his hypothesis of inversion in the hydrolysis of sugar phosphate in nature is correct it may well be difficult to find the conditions for the imitation of the process in the laboratory, and secondly, that the glucose-galactose change involves position 4 where the stereochemical conditions are different from those in the glucose-3-phosphoric acid used by Levene.

Levene *et al.* [1929] found, in favour of Robinson's suggestion, that the hydrolysis of the phosphoric group in fructose-3-phosphoric acid during osazone formation is accompanied by a Walden inversion.

It may be remarked that the reserve polysaccharide of fenugreek seeds, a detailed study of the behaviour of which is provided in this communication, is a mannogalactan which is also esterified by phosphoric and silicic acids. On germination it gives rise to glucose and fructose, while, apart from the seeds, hydrolysis by different means results only in the production of galactose and mannose. A hydrolysis of the original ester takes place in all cases.

Mathers and Robertson [1933], working on the hydrolysis of *p*-toluenesulphonyl esters of glucose, were able to convert a derivative of glucose into a derivative of altrose. Cognate researches by Robertson and Oldham [1934] provided evidence that the hydrolytic reactions fall into two main groups, normal and abnormal, according as the original substance contains one or two *p*-toluenesulphonyl residues in neighbouring positions. They have also shown that in cases in which Walden inversion has been proved to occur, such inversion is accompanied by anhydro-formation. According to them the anhydro-formation may be a necessary precursor of this type of inversion, which follows as a consequence of the opening of the anhydro-ring.

Lohmann [1933] has shown that in yeast and animal cellular extracts there is an agent which converts both ketohexose- (Neuberg ester) and aldohexosemonophosphoric ester into Embden ester which is an equilibrium mixture composed of aldo- and keto-hexosemonophosphoric esters in which the former are present to a predominant extent. It may be noted that this investigation applies only to the transformation of an aldose into its ketose analogue or *vice versa.*

Robison [1932] and Macleod and Robison [1933], working on the products of yeast fermentation and on the hydrolysis of hexosediphosphate produced during the fermentation, suggest schemes which may possibly apply to the transformation of one hexose into another. According to these schemes an enolic compound common to glucose, fructose and mannose is formed, which reacts with phosphoric acid and the resulting enolic phosphate passes into a mixture of esters of the three above sugars. But, again, these schemes suggest only the mechanism of the interconversion of a sugar with its epimeride and its ketose analogue and, if applied to the problem under investigation as a whole, they give no clue to the transformation of a sugar like galactose into glucose or fructose as happens during the germination of seeds.

EXPERIMENTAL.

It has already been mentioned that the sugars entering into the constitution of the reserve polysaccharide of fenugreek seeds are mannose and galactose, which are transformed during germination into glucose, fructose and sucrose. It is natural to attribute such transformations to the influence of enzymes elaborated by the seeds. As the transformation may be brought about by the co-operation of more than one enzyme, it was decided to study the action of the enzymic mixture of the germinated seeds on the polysaccharide *in vitro.*

The enzyme preparations.

Fenugreek seeds, in different stages of germination, were used for the preparation of the enzyme mixture. The seeds were first minced in a meat mincer and ground up in a mechanical mortar into a fine paste, which was then extracted either with chloroform water or with glycerol. The proportion of solvent in each case was 1 ml. per g. of paste. The paste was allowed to stand with either solvent for different intervals of time varying from 6 hours to 10 days and was shaken from time to time. For some experiments the paste was treated with distilled water and was mechanically shaken for 2½ hours after which it was prepared for immediate use. For use the mixture of paste and any of the solvents used was placed in a calico bag and pressed out by hand, followed by a press. The liquid obtained by centrifuging the fluid was used in the experiments. In some experiments the enzyme mixture used was prepared by treating the paste with a small amount of water and a little toluene and then allowing to stand for a few days in an incubator in order to allow the cells to autolyse and to liberate their enzymes. Water was then added to the mass which was subsequently treated as above.

Action of the mixture of enzymes on the polysaccharide in vitro.

It should be remembered that the mixture of enzymes prepared by any of the above methods contains, in case of short extractions, glucose, fructose and sucrose. After prolonged extraction the amount of sucrose diminishes, finally leaving only the two former sugars. This is due to the sucrose undergoing hydrolysis by the invertase which is present in the extract. The preparations also contain the polysaccharide which had not been utilised during germination, and also, in the case of long extractions, its hydrolytic products.

The quantity of the enzymic solution employed was usually 2–3 ml. for every 40 ml. of the polysaccharide solution; thus the amount of the sugars originally present in the reaction mixture due to the enzymic preparations was too small to influence the identification of the sugars resulting from the hydrolysis of the polysaccharide. Nevertheless, controls with the same quantity of enzyme solution added to a quantity of water equivalent to the volume of the polysaccharide solution used were performed at the same time and treated in a similar manner.

The purified polysaccharide of fenugreek seeds, which is a silicophosphoric ester of mannogalactan, was prepared by the method of Daoud [1932]. Different antiseptics (toluene, camphor, thymol, chloroform and sodium fluoride) were used in the different experiments.

When the mixture of enzymes, prepared by any of the above-mentioned methods, was added to the polysaccharide solution together with one of the antiseptics and incubated at 37°, it was noticed that the opalescence as well as the viscosity of the solution gradually diminished with simultaneous deposition of an insoluble residue, while the reducing power gradually increased. The insoluble residue was found to be composed mostly of inorganic constituents including silica, phosphates, iron, calcium and magnesium. Portions of the reaction mixture, removed at different intervals of time, were treated with an equal volume of 95 % alcohol; the precipitate of polysaccharide obtained showed a gradual diminution in quantity and gradual changes in property as the reaction proceeded. The precipitate obtained as soon as the enzyme was added was a white filamentous mass, while that obtained at advanced stages of the reaction was white and pulverulent and dissolved in water with greater ease.

The changes taking place during the action of the mixture of enzymes appear to be as follows: firstly, there is a hydrolytic removal of the silicic and phosphoric acids, which is most probably brought about by esterases with the production of a more soluble substance, and secondly, a simultaneous or subsequent hydrolysis of the de-esterified substance into sugars. There is also the possibility that the more soluble substance includes degradation products of the original polysaccharide. The de-esterification proceeds at a greater rate than the hydrolysis into sugars since the precipitate of silica and phosphates produced during the enzymic action ceases to increase after a certain period while the reducing power continues to increase further.

To investigate the nature of the sugars produced by the action of the enzymes on the polysaccharide, the following procedure is adopted.

The reducing power is determined from time to time by removing portions of 25 ml. of the reaction mixture, making up to 50 ml. with 95 % alcohol, filtering, washing with 50 % alcohol, evaporating the alcohol, making up the residue to the original volume with water and determining the amount of reducing sugars by Bertrand's method.

When the reducing power shows little or no further increase, the whole of the reaction mixture is removed from the incubator, filtered from the insoluble residue, shaken with a small amount of calcium carbonate and refiltered. The filtrate is treated with alcohol to precipitate any traces of polysaccharide which sometimes remain unattacked, refiltered if necessary and finally concentrated in a vacuum to a smaller volume than the original. This somewhat concentrated solution is treated with normal lead acetate until maximum precipitation is reached, any excess of lead being removed by sodium carbonate; the filtrate after removal of the lead is neutralised with acetic acid and decolorised.

When portions of the solutions thus obtained are heated with phenylhydrazine hydrochloride and sodium acetate, two osazones are obtained, one of which is identical with glucosazone and the other with galactosazone. The presence of galactose is further confirmed by the production of mucic acid on oxidising a portion of the solution with nitric acid. On treating other portions of the solutions with phenylhydrazine and acetic acid in the cold a white crystalline precipitate is obtained which is identified by its crystalline form and its melting-point as mannosephenylhydrazone. The glucosazone obtained on heating, with the exception of a very small amount which is due to the glucose and fructose content of the enzyme solution, is entirely due to mannose and not to glucose or fructose which might have been formed during the breakdown of the polysaccharide. This is proved by adding an excess of phenylhydrazine dissolved in acetic acid to a portion of the cold solution and keeping the mixture in the ice-chest for 24 hours, then filtering off the mannosephenylhydrazone, heating the filtrate in a water-bath, and examining the osazone thus formed after the removal of the mannose. It is found that the main osazone present is galactosazone together with a very small amount of glucosazone which is as above-mentioned due to the sugars of the enzyme solution. Further, quantitative experiments prove beyond any doubt that the sugars produced by the enzymic action on the polysaccharide are entirely mannose and galactose. These quantitative experiments are performed as follows.

A measured volume of the polysaccharide solution is treated with a known volume of one of the enzymic preparations. At the same time a control is made by the addition of the same volume of the same enzymic preparation to a volume of water which is equal to the volume of the polysaccharide solution employed. Both solutions are incubated till no precipitate of polysaccharide is produced on the addition of alcohol to a portion of the polysaccharide solution and they are then filtered and equal volumes of the filtrates are clarified and decolorised, taking equal measured volumes of the filtrates after each process. The mannose in the final solutions is estimated as mannosephenylhydrazone. After allowing for the quantity of mannose in the control, it is found that in all cases the yield of mannose from the polysaccharide alone by the action of the enzyme almost agrees with the theoretical amount of mannose which is calculated to be present in the quantity of polysaccharide originally taken. The amount of mannose found is never less than 98·8 % of the theoretical amount.

The above solutions are also used for calculating the specific rotatory power of the mixture of sugars resulting from the enzymic hydrolysis of the polysaccharide. The percentage of reducing sugars calculated as glucose and also the rotation of the final solution in the case of the control are subtracted from those of the final solution derived from the polysaccharide. The balance of the rotation is considered to be due to the amount of sugars resulting from the enzymic action on the polysaccharide only. The specific rotatory power calculated on this assumption for the several experiments performed varies from $[\alpha]_D^{20^\circ} + 45{\cdot}9^\circ$ to $[\alpha]_D^{20^\circ} + 48{\cdot}1^\circ$. The theoretical specific rotatory power for a mixture of equal quantities of galactose and mannose is $[\alpha]_D^{20^\circ} + 47{\cdot}5^\circ$.

Since the yield of mannose from the polysaccharide, which contains equal quantities of mannose and galactose, is quantitative, and these two sugars are produced by the enzymic action in equal quantities, it follows that the only products of the enzymic action on the polysaccharide are galactose and mannose.

It was noticed that the extracts of the germinated seeds were usually slightly acid to litmus. This observation suggested that the hydrogen ion concentration might have an effect in causing the interconversion of the hexoses to take place in the germinating seeds. The polysaccharide solution was buffered at p_{H} values varying between 5·4 and 7·8. It was found that the production of galactose and mannose from the polysaccharide by the action of the enzymic mixture is independent of the hydrogen ion concentration of the medium within these limits.

The above observations lead to the conclusion that the hydrolytic products of the reserve polysaccharide of fenugreek seeds are always the same whether the hydrolysis is effected by acids or by the enzyme mixture from the germinated seeds *in vitro*.

Direct investigations on the germinating seeds.

It was noticed that while mature ungerminated fenugreek seeds are usually starch-free, the germinated ones contain starch and that this increases in amount with the progress of germination. Some specimens of fenugreek seeds were found to contain traces of starch before germination; this, however, is rare and is most probably due to an immaturity of the seeds or an imperfect drying and preservation of the harvest. It was proved by various methods that the substance formed during germination is starch. In some cases the starch appeared to have an admixture of some other substance probably of the nature of an erythrodextrin, since on the addition of iodine to the isolated substance a violet coloration instead of blue was sometimes given.

The starch formed was not the result of photosynthesis as in all cases the investigations were made before the cotyledons left the testa or showed any sign of chlorophyll formation. Furthermore the steeping and the germination were carried out in darkness.

The importance of determining the relation of the sugars, especially that of glucose, appearing during germination to the starch is apparent. An attempt was therefore made to find out which of these compounds first appears in the germinating seeds. This presents certain difficulties since in most seeds starch and sugars seem to occur practically at the same time at the beginning of the germination. On carefully following the formation of starch in starch-free fenugreek seeds, it was observed that this substance appears during steeping; usually after 10 to 18 hours of steeping. The following procedure was therefore adopted to find out which of the above substances appears first.

A weighed quantity of the seeds steeped till the starch became detectable was thrown into a small volume of vigorously boiling water to stop all enzymic action. Boiling was continued for one hour. The seeds were then drained, weighed, ground into a paste and remixed with the drained water. Alcohol was added to this mixture to a concentration of about 50 %, taking into consideration the quantity of water absorbed by the seeds during the steeping. Calcium carbonate was added to neutralise the solution which was then boiled on a water-bath under reflux for 2 hours in order to extract any sugars formed. The filtered extract was distilled *in vacuo* to a small bulk. The concentrated residue was clarified and examined for reducing sugars by the osazone test. In the great majority of the experiments no osazone could be detected. In very few exceptional cases small traces of glucosazone were found. The amount was exceedingly small if it be remembered that we dealt in each case with a concentrated extract of 100 ml. obtained from 1 kg. of the dry seeds. This result indicates that the formation of starch precedes that of glucose. It has been observed that while the reserve mannogalactan of fenugreek seeds is contained in the endosperm, the formation of starch takes place entirely in the cotyledons and radicles. The amount of starch in these organs increases with the exhaustion of the endosperm, thus indicating the formation of starch at the expense of the reserve mannogalactan.

Further support of the view that the starch formation precedes that of the glucose was gained by observations made on single germinating seeds of carob (*Ceratonia Siliqua*). The reserve polysaccharide of these seeds is also a mannogalactan which, as in the case of fenugreek seeds, gives glucose, fructose and sucrose during germination. These seeds are difficult to germinate under laboratory conditions as they possess a very hard testa and a thick hard endosperm, while the cotyledons are very thin and practically free from carbohydrates. The straight radicles of these seeds allowed easy measurement which helped in determining the beginning of germination. Germination was only possible after steeping for a long time and then carefully removing the testa and the mucilage layer round the radicle. The germination was still slow even after exposing the radicles. In order to have the cotyledons accessible for the purpose of detecting the first appearance of starch, the testa at the top of the seeds was removed. The seeds were always kept moist during germination. Every 12 hours a minute fragment of one of the cotyledons was removed at the top of the seed by means of a thin needle and tested with iodine under the microscope. It was found that starch makes its appearance in the cotyledons before the radicles show any increase in length. Elongation of the radicles was almost simultaneous with the appearance of reducing substances in them; both take place between 24 and 36 hours after the first appearance of starch. It was further noticed that some seeds failed to form starch and these never germinated.

It is, therefore, justifiable to assume that the mannogalactan of carob and fenugreek seeds gives rise to starch and that the glucose which appears during germination arises as a result of hydrolysis of the starch by an amylase which has been experimentally found to be elaborated during germination.

The experiments just described gave a clue to the probable course followed in the formation of the reducing sugars found in the radicles of the germinating seeds, sugars which are entirely different from those constituting the reserve. If the starch supplies the glucose, another compound might be expected to be responsible for the supply of fructose. Other polysaccharides were, therefore, looked for. The concentrated clarified extracts obtained from the steeped fenugreek seeds by the method described before were treated with alcohol. A precipitate began to appear when the concentration of alcohol was about 64 % and gradually increased with the addition of more alcohol till the concentration reached 75 %. The small precipitate formed was collected by centrifuging, redissolved and reprecipitated three times, treated with absolute alcohol followed by ether and dried *in vacuo*. The white powder so obtained was readily soluble in water, did not reduce Fehling's solution and was, in most cases, laevorotatory. In a few cases, however, the substance had a feeble dextrorotation. The variation in rotation suggested a non-homogeneous constitution for this substance. The product was, therefore, subjected to fractional precipitation. It was found that a portion came down when the concentration of alcohol was about 64 %. This was collected by centrifuging and proved to be a pentosan. When the concentration of alcohol in the remaining fluid was raised to 70–75 % a second minute fraction was obtained. This fraction was white when dry, tasteless, did not reduce Fehling's solution and was laevorotatory. When hydrolysed with 1 % sulphuric acid under reflux on a boiling water-bath for half an hour, the hydrolysate after neutralisation with barium carbonate and filtration reduced Fehling's solution, gave an osazone identical with glucosazone, yielded the Pinoff-Seliwanoff's reaction and had a laevorotation. The specific rotation of the hydrolysates obtained from different experiments varied from $[\alpha]_D^{20^\circ} -89{\cdot}7^\circ$ to $-91{\cdot}2^\circ$. The specific rotation of fructose is $[\alpha]_D^{20^\circ} -92{\cdot}5^\circ$.

This clearly indicates that the sugar obtained on the hydrolysis of the above fraction is fructose, and that the original substance is composed of polymerised fructose residues and is similar to, if not identical with, inulin. It will be referred to as fructosan.

After removal of the fructosan, the fluid still contained a substance of a carbohydrate nature which came down only when the concentration of alcohol was very high. This rendered its separation by this method inconvenient. This substance was found to form a precipitate with Fehling's solution which was used for its separation. The alcohol contained in the fluid after the removal of the two above-mentioned fractions was distilled and Fehling's solution added. The plastic precipitate formed was rapidly collected, dissolved in a little dilute hydrochloric acid and precipitated with acetone. The precipitate was collected, redissolved, reprecipitated with acetone three times and finally dried. The dried substance is pale-yellow in colour, readily soluble in water with frothing, it has a bitter taste and causes a slight reduction of Fehling's solution when heated or in the cold if allowed to stand for a sufficient time; it gives no osazone and is laevorotatory. When its solution is hydrolysed, it shows a dextrorotation, its reducing power greatly increases, while a considerable amount of precipitate is formed. This shows that the substance behaves in every respect like a saponin; further it gives similar colour reactions and causes haemolysis.

As this saponin was found to be present in the dry ungerminated fenugreek seeds and as it was proved to be utilised during germination, it became necessary to determine the nature of its sugar content. On hydrolysis galactose and mannose were the only two hexoses identified.

All the above experiments show that the reserve carbohydrates of fenugreek seeds are entirely constituted of mannose and galactose and that the whole glucose and fructose which appear during germination are to be regarded as transformation products.

The identification of the minute amount of fructosan in the steeped seeds necessitated the examination of the ungerminated seeds for the presence of that substance. Powdered ungerminated fenugreek seeds were extracted with 50 % alcohol following the steps already mentioned in the extraction of the steeped seeds. When the clarified solution was treated with alcohol, the precipitate obtained between 64 and 75 % concentration was in all cases dextrorotatory.

When alcohol was added to the clarified solution to a concentration of 75 % the precipitate obtained, contrary to the case of the steeped seeds, could not be fractionated into a pentosan and a laevorotatory body. Pentoses were the only products obtained on hydrolysis. This shows that the fructosan is not originally present in the ungerminated seeds, but that, similarly to starch, it is formed only during steeping.

Examination of the seeds in different conditions for the presence of an enzyme which attacks inulin has shown that while extracts of the ungerminated seeds have no action on that substance, those of the steeped and germinated feebly attack it. The secretion of an enzyme which attacks inulin simultaneously with the formation of the fructosan in the steeped or germinated seeds suggests that the fructosan formed is built up on lines similar to inulin.

Since no glucose or fructose is formed in a free state during the period of steeping, it becomes clear that both the starch and the fructosan formation from the galactose and the mannose constituting the reserve polysaccharide of the ungerminated seeds must precede the appearance of glucose and fructose of the germinated seeds. This explains the failure of the enzymic mixture of the germinated seeds to produce the interconversion of the hexoses of the reserve polysaccharide *in vitro*.

Having demonstrated that starch and fructosan appear in the steeped seeds before the appearance of reducing sugars, the behaviour of these two polysaccharides was next studied during different stages of germination of the fenugreek and other seeds. The results obtained can be summarised as follows. On germination the starch gradually increases in amount; the fructosan can be detected in fenugreek seeds during all stages of germination but always in small quantities, it seems to diminish further or to disappear on prolonged germination. In carob seeds the fructosan could not be detected at all. The following table illustrates the changes in the quantities of the starch and the fructosan during different stages of germination of fenugreek seeds. The figures are expressed in g. per 100 g. of the dry seeds.

Exp.	Time	Starch	Fructosan
I	Before steeping	0·00	0·00
	1 day germination	1·48	0·067
	2 days ,,	2·44	0·071
	4 ,, ,,	4·15	0·054
	6 ,, ,,	5·97	0·035
	8 ,, ,,	7·71	0·021
II	8 ,, ,,	8·02	Practically nil
III	8 ,, ,,	8·31	0·014

It has already been mentioned that, besides the glucose and the fructose, sucrose is also formed during the germination of fenugreek seeds. Examination of seeds similar to those of fenugreek as regards their reserve polysaccharides and of other seeds containing different forms of reserve polysaccharides, such as galacto-araban and starch, proved that sucrose is always produced during germination. Experiments to show the relation between sucrose and the reducing sugars glucose and fructose were performed by quantitatively determining the amounts of these sugars at the early stages of germination in the different seeds examined. This was done by extracting the three sugars with boiling 80 % alcohol, taking all the precautions already mentioned in investigating the relation between starch and glucose. The results of these experiments as well as the significance of sucrose and the part it possibly plays in the mechanism of the interconversion of the hexoses will be referred to in the discussion.

DISCUSSION.

It is clear from the foregoing experiments that formation of starch and a fructosan from the galactose and the mannose of the ungerminated seeds precedes the appearance of glucose and fructose in the germinated seeds. It would appear therefore that the transformation of one hexose into another occurs through an intermediate formation of a polysaccharide which gives rise to the new sugar on hydrolysis. When the radicles of dry non-starchy seeds are removed and the residual parts of the seeds are steeped and incubated at the same time with similarly treated seeds but with radicles left intact, neither starch nor sugars appear in the former while considerable amounts of both appear in the latter.

The work of Rohmann [1919] affords further evidence that in the process of interconversion there is an intermediate polysaccharide formed which gives rise to the new sugar. He found that the mammary gland contains a substance not identical with glycogen from which lactose may be obtained by ferment action. He points out that this substance is formed in the milk gland out of *d*-glucose and that on hydrolysis of this complex substance lactose is produced. It follows that the galactose constituent of the lactose molecule must arise from *d*-glucose during the polysaccharide formation.

Taking into consideration the work of the different investigators, including the present work, it can be concluded that this mechanism, if not universal amongst living organisms, plays at least a conspicuous part in the interconversion of hexoses.

Thus, according to this point of view, the actual transformation of one sugar into another takes place during the polymerisation of the hexose residues of the sugar undergoing transformation to form the polysaccharide which ultimately gives rise to the new sugar. That the process does not involve a direct transformation of one polysaccharide into another is deduced, firstly, from the presence of a powerful enzyme in the seeds which hydrolyses the original polysaccharide into its constituents and, secondly, from the fact that the starch formation in endospermic seeds takes place in the cotyledons and radicles but not in the endosperm where the original polysaccharide is present. Since the diffusion of colloidal matter through cell membranes is not likely to take place it is more probable that it is the constituent sugars of the original polysaccharide which diffuse into the embryo and polymerise to form the new polysaccharide. In order to see how this agrees with the well-known structural and other characteristics of sugars and polysaccharides, the following may be brought into consideration.

The difference between galactose and glucose lies in the configuration of carbon atom 4 and it has been shown by Haworth and Percival [1931] that in starch, the glucose residues are linked together by an α-linkage joining the fourth carbon atom of one residue with the terminal carbon atom 1 of another residue. It is seen, therefore, that it is the same carbon atom 4, the configuration of which determines the difference between galactose and glucose, which is concerned in the linkage of the hexose residues. This indicates the possibility of the stereoisomeric change taking place on the polymerisation of the residues to form a polysaccharide.

In the transformation of a sugar like mannose or glucose into fructose, one hydrogen atom on carbon atom 2 must change position with the oxide ring on carbon atom 1. In inulin, the mode of linkage of the fructose residues has been determined by Drew and Haworth [1928]. According to these authors the junction of pairs is between the reducing group 2 of one residue and the terminal group 1 of another residue, while the oxide ring connects positions 2 and 5 in each residue; thus according to this inulin is composed of γ-fructose residues. In view of the appearance of the enzyme inulase in the germinating seeds of fenugreek, the fructosan formed during germination is supposed to be built up on similar lines to inulin. Whatever the constitution of the fructosan may be, it is evident that in a non-reducing polysaccharide composed of fructose residues the reducing group 2 must be concerned in the linkage of residues. It is seen, again, that the carbon atom concerned in the linkage of the residues is the atom at which the rearrangement takes place, and it is quite possible that such a rearrangement between a hydrogen on carbon atom 2 and the oxide ring on carbon atom 1 in glucose or mannose is effected by the linkage of carbon atom 2 to another hexose residue on polymerisation of the residues for the formation of the fructosan.

It may be remarked that the fructosan formed during the steeping and the germination of fenugreek seeds is either a highly labile substance or a substance of no great importance for the mechanism of fructose formation since, contrary to starch, it is only found in small quantities at any stage of germination. Further the experiments show that on prolonged germination the fructosan diminishes considerably or even vanishes, and that in seeds of similar reserve, as well as some seeds of different forms of reserve, it could not be detected. On the other hand sucrose is formed in considerable amounts during the germination of seeds containing different forms of reserve polysaccharides. In early stages of germination of seeds in which the fructosan was not identified it has been found that when the reducing sugars just become detectable the amount of sucrose is considerably greater than that of reducing sugars. Had the sucrose been formed at the expense of the reducing sugars glucose and fructose such a result would not be expected. Further, with the production of sucrose there is also a production of a strong invertase. These observations indicate the possibility of the sucrose playing a part in the mechanism of fructose formation. The fructose residue of sucrose can arise from mannose or from glucose on condensation with another glucose residue in a manner similar to that described for the fructosan formation. Again Haworth [1920] has shown that the linkage between fructose and glucose in sucrose is through their reducing groups, the fructose residue being present in the γ-form, *i.e.* in the formation of sucrose the carbon atom 2 of one hexose residue is concerned in the condensation.

The following two possibilities of the transformation of a hexose into a polysaccharide which subsequently gives rise to another hexose suggest themselves:

(1) A stereoisomeric change which takes place at the carbon atom concerned in the linkage of the hexose residues as, for example, the transformation

of galactose into glucose through starch or *vice versa*, *i.e.* glucose into galactose through a polymeride like the reserve polysaccharide of some seeds.

(2) Intramolecular rearrangement between a hydrogen atom on carbon atom 2 and the oxide ring on carbon atom 1 in the same residue. This takes place in the transformation of an aldose into its ketose analogue as, for example, in the transformation of glucose or mannose into fructose through a polymeride of the latter or its condensation product with another hexose forming a higher sugar. The reverse rearrangement may take place in the transformation of fructose into mannose or glucose through polysaccharides like starch, glycogen or compounds containing mannose.

The possibility of the transformation of aldoses into polymerides of ketoses has been indicated by Colin [1918], who showed that inulin was formed in places of storage by condensation of the sugars synthesised by the leaf.

Evidence of the transformation of fructose into glucose through the intermediate formation of glycogen can be gained from the work of several investigators, *e.g.* Wittgenstein [1923]; Cori [1926]; Cori and Cori [1928] and Corley [1929]. The work of these authors leads to the conclusion that fructose requires transformation into glycogen before it can be utilised. In the formation of glycogen, of starch and generally of non-reducing polysaccharides composed of aldose units, the junction of pairs of units must be through the terminal reducing group of one residue and some other group in the other residue. It is, again, clear that the carbon atom 1 which is concerned in the junction of units is the atom at which the rearrangement takes place between the oxide ring on carbon atom 2 of fructose and a hydrogen atom on carbon atom 1. The transference of the hydrogen atom to carbon atom 2 may lead to the production of either glucose or mannose residues in the resultant polysaccharide. This assumption postulates that in the transformation of glucose or mannose into fructose or *vice versa* the oxide ring in either case moves to the carbon atom concerned in the linkage of pairs of units.

The consideration of the structure of the fructose residue in the natural plant products gives further support to these views. It can be concluded from recent investigations that fructose is the only sugar known to exist in the γ-form in its polymeride inulin and its condensation products like sucrose. According to the mechanism under discussion, when a sugar like glucose or mannose is transformed into a polymeride of fructose or a condensation product of it with another sugar, the ring which is an amylene oxide ring in the original sugar, *i.e.* joins positions 1 and 5, will now join positions 2 and 5, *i.e.* becomes a butylene oxide ring. This may give an explanation of the natural existence of fructose in the γ-form in its polymerides or condensation products only.

It remains to explain, on similar grounds, the transformation of mannose into glucose. During the present work evidence has been obtained that such a transformation takes place. The only carbohydrate reserve of carob seeds (*Ceratonia Siliqua*) is a polysaccharide composed of mannose and galactose similar to that of fenugreek seeds, but it has been found that the two sugars exist in the proportion of four of the former to one of the latter, while in fenugreek seeds the two exist in equal quantities. When the seeds were germinated, glucose, fructose, sucrose and starch were formed. Examination of the radicles proved that the glucose and the fructose existed in almost equivalent quantities; the sucrose also contains these two constituents in equivalent quantities and in addition to that, starch is formed which is composed entirely of glucose residues. If we consider that the glucose arises from galactose only, the glucose content of the germinated seeds would not exceed one-fifth of the total hexose content of the different above-mentioned compounds. The fact that the glucose element is in excess over the fructose one can only be explained if we assume that mannose also gives rise to glucose.

Applying the mechanism under discussion to the transformation of mannose into glucose, we must consider that starch in the higher plants and glycogen in the lower plants and animals are intermediate compounds. The difference between mannose and glucose lies in the configuration of the second group of the hexose molecule; but in the formation of a compound like starch from hexose residues the fourth carbon atom is the one concerned in the linkage of pairs. Unless we know more about the nature of the polysaccharides and the mode of polymerisation no explanation can be given with certainty, but two possibilities suggest themselves.

(1) A formation of an intermediate polysaccharide composed of glucose residues in which the junction of pairs is at the carbon atom 2 of one residue and the terminal carbon atom 1 of another residue. Such a polysaccharide has not been identified in the steeped or germinated seeds.

(2) A more probable explanation which fits to some extent with experimental facts is that the mannose is transformed into a fructosan or gives rise to a fructose residue on condensation with another hexose forming a non-reducing reserve sugar such as sucrose. These fructose-containing compounds give rise to fructose which undergoes polymerisation forming a glucose precursor such as starch or glycogen. This assumption is supported by the observation already mentioned that the fructosan found in steeped and germinated fenugreek seeds always occurs in small quantities and further diminishes or even vanishes with prolonged germination. Secondly, in all non-starchy germinated seeds the glucose element in its different forms, especially starch, is found in considerable excess over the fructose element. These observations tend to show the transformation of fructose and its compounds into starch. In this respect, it is of interest to mention that Colin and Belval [1922; 1923] working on the genesis of carbohydrates in wheat, found that a fructosan is present in the stem during the flowering season and in the seeds a little after that season. This fructosan gradually disappears with the maturity of the seeds, thus indicating the formation of starch at the expense of this compound.

SUMMARY.

1. An important mechanism of the isomeric change of one hexose sugar into another in the living organism is the formation of an intermediate polysaccharide or a condensation product such as a non-reducing sugar which gives rise to the new sugar on hydrolysis.

2. It is suggested that the actual transformation of the sugar takes place on polymerisation or condensation in one of two ways. Either a reversal of the stereochemical configuration of the carbon atom concerned in the linkage of pairs of the hexose residues, or an intramolecular rearrangement between the oxide ring and a hydrogen atom on the first and second carbon atoms of the hexose molecule. The oxide ring moves to the carbon atom concerned in the linkage of pairs of residues. The former case occurs in the transformation of a sugar into its stereoisomeride, *e.g.* galactose into glucose or *vice versa*, and the latter case during the transformation of a sugar into its ketose analogue, *e.g.* glucose or mannose into fructose or *vice versa*.

3. An explanation of the natural existence of fructose in the γ-form in its polymerides such as inulin or its condensation products such as sucrose can be

given on the basis of these views. It is assumed that such plant products originate from aldoses, such as glucose, of the photosynthetic process on polymerisation or condensation.

4. The influence of the living organism is a determining factor in the process of interconversion since the elaboration of the intermediate polysaccharides does not take place in the absence of the organism. When the enzymic extracts of germinated seeds in which interconversion of the hexoses takes place are allowed to act on the reserves of these seeds *in vitro*, the end products obtained are identical with those obtained by acid hydrolysis.

If the vitality of the seeds is destroyed by removing the radicles, then on steeping and incubating the seeds, no intermediate products or sugars are formed.

5. Together with the production of the intermediate compounds in seeds there is also a production of the specific enzymes which attack them.

6. Starch, besides playing a part in the mechanism of the interconversion of the hexoses, is usually the principal reserve carbohydrate of seeds. In starch-free seeds the different forms of carbohydrate reserves are transformed during germination mainly into starch. The other intermediate compounds playing a part in the interconversion of hexoses are formed in much smaller amounts than starch and are possibly transformed into it. The process is reversible and starch in its turn can give rise to other forms of reserves when necessary.

In starch-free endospermic seeds the starch formation takes place in the cotyledons and radicles at the onset of metabolic processes but not in the endosperm where the original reserve carbohydrates mainly exist.

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REFERENCES.

Colin (1918). *Compt. Rend. Acad. Sci.* **166**, 224, 305.
—— and Belval (1922). *Compt. Rend. Acad. Sci.* **175**, 1441.
—— —— (1923). *Compt. Rend. Acad. Sci.* **177**, 343.
Cori (1926). *Proc. Soc. Exp. Biol. Med.* **23**, 459.
—— and Cori (1928). *J. Biol. Chem.* **76**, 755.
Corley (1929). *J. Biol. Chem.* **81**, 81.
Daoud (1932). *Biochem. J.* **26**, 255.
Drew and Haworth (1928). *J. Chem. Soc.* 2690.
Haworth (1920). *J. Chem. Soc.* 199.
—— and Percival (1931). *J. Chem. Soc.* 1342.
Levene (1927). *Nature*, **120**, 621.
—— Raymond and Walti (1929). *J. Biol. Chem.* **82**, 191.
Lohmann (1933). *Biochem. Z.* **262**, 137.
Macleod and Robison (1933). *Biochem. J.* **27**, 286.
Mathers and Robertson (1933). *J. Chem. Soc.* 1076.
Robertson and Oldham (1934). *Nature*, **133**, 871.
Robinson (1927, 1). *Nature*, **120**, 44.
—— (1927, 2). *Nature*, **120**, 656.
Robison (1932). *Biochem. J.* **26**, 2191.
Rohmann (1919). *Biochem. Z.* **93**, 237.
Wittgenstein (1923). *Deutsch. med. Woch.* **49**, 246.

XXVIII. PROBLEMS OF NITROGEN CATABOLISM IN INVERTEBRATES.

II. CORRELATION BETWEEN URICOTELIC METABOLISM AND HABITAT IN THE PHYLUM *MOLLUSCA*.

By JOSEPH NEEDHAM.

From the Biochemical Department, Cambridge, and the Marine Biological Laboratory, Plymouth.

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In the preceding paper of this series [Baldwin and Needham, 1934] some figures were reported for the uric acid content of the nephridium or kidney of the large land-snail, *Helix pomatia*. It was found that even for a considerable time after the end of the hibernation period, no less than three-quarters of the dry weight of the organ could consist of uric acid. This remarkable retention stimulated a desire to investigate whether other gastropod nephridia showed a like accumulation, and the possibility arose of studying in this way the distribution of uricotelic metabolism. We are accustomed to the view that the use of uric acid as the main vehicle of waste nitrogen excretion may in some way or other be regarded as an adaptation to terrestrial life, and the suggestion has been made [Needham, 1929; 1931; see also Hopkins, 1928] that terrestrial oviparity in particular, where development within the cleidoic egg occurs, is associated with uricotelic metabolism. Hitherto, however, such generalisations have had perforce to rest upon our knowledge of the nitrogen metabolism of vertebrates only. The urea production of fishes, amphibia and mammals has been compared with the uric acid production of reptiles and birds. But the invertebrates may perhaps provide a still more striking opportunity for the correlation of metabolism with habitat. In the insects, where the excretion of uric acid as the main end-product is widespread if not universal, such opportunities, it is true, are not promising, for the Subphylum Insecta is as a whole so typically terrestrial, and the aquatic forms are so obviously secondary, that the boundaries of habitat and metabolism will be the boundaries of the group. In the molluscs, however, we have a morphologically homogeneous population distributed through every conceivable environment; thus the gastropods are to be found in the sea and on its littoral, in brackish-water and fresh water, both rivers and lakes, and on land in surroundings showing every degree of humidity. The lamellibranchs, on the contrary, are mainly marine, with a few fresh-water species. Amphineura, Scaphopoda, and Cephalopoda are wholly marine. With the probability that terrestrial snails are uricotelic organisms now raised almost a certainty [see Baldwin and Needham, 1934] we are evidently at liberty to enquire how far this form of metabolism extends and to correlate within one group of genera the environment and the metabolism.

Strohl [1914, p. 527], in his valuable review of the excretory mechanisms of molluscs, concludes that in general the gastropods are characterised by positive findings of uric acid in their nephridia, but that in the lamellibranchs this substance is always absent, and its place seems to be taken by urea. This statement, it will be seen, is in rough agreement with the expectations which we derive from facts mentioned in the preceding paragraph. But it rests entirely on the qualitative tests scattered through the literature (which he fully cites). From these it is difficult to draw very far-reaching conclusions, if only for the reason that a positive uric acid test might imply simply the presence of a nuclein metabolism similar to that of mammals and nothing whatever regarding the excretion of the main portion of the waste nitrogen as uric acid. Moreover, it must be admitted that many of the workers reviewed by Strohl employed techniques which would not receive unqualified approval to-day. This vitiates any wide conclusions drawn from the general body of their work.

There are two main ways in which uricotelic metabolism could be proved to exist in molluscs, firstly, by the direct estimation of the various nitrogenous substances excreted by them and the drawing up of a table of nitrogen partition, secondly by the demonstration of the synthesis of uric acid from ammonia by tissue-slices *in vitro*. Unfortunately, both these methods suffer from difficulties, some of which are special to the molluscs. The direct estimation of the end-products, though straightforward enough in the case of terrestrial or fresh-water species, presents technical difficulties if the animals have to be kept in sea-water, especially if a high degree of accuracy is desired. Worse than this, however, is the very erratic nature of the excretion of many species and the possibility that others, although breaking down protein with the ultimate formation of uric acid, never excrete the latter at all. Thus experience in the cultivation of *Helix pomatia* shows us that an emptying of the nephridial contents, with the formation of clay-like granular masses, does not take place more often than once a fortnight or three weeks. Other snails probably excrete more rarely [Simroth and Hoffmann, 1928]. *Cyclostoma elegans*, the terrestrial operculate, possesses a special dermal gland (referred to later) in which uric acid is stored in extremely large amounts, and nothing is known about its excretion. In such a case, the measurement of the types of substance excreted might be very misleading indeed in the absence of other information even if carried on over very long periods. Then with regard to the demonstration of the synthesis of uric acid from ammonia by tissue-slices, difficulties arise owing to the relative slowness of the nitrogen metabolism in gastropods, leading to unduly long experiments. Here other limitations such as the tendency of the hepatopancreas, for instance, to fragmentation, and the danger of bacterial attack, are soon met with. Much further research, too, will be needed before we know the optimum requirements for invertebrate tissues *in vitro*, and there may be a poisoning of the synthesising cells by ammonia. Finally the smallness of many of the most interesting molluscs makes the study of their tissues *in vitro* for the present as good as impossible.

In view of all these considerations, it was decided to attempt a preliminary survey of the molluscs upon the assumption that the existence of uricotelic metabolism in any particular case would be reflected in the degree of retention of uric acid by the nephridium, or what might be called the average load, and to examine accordingly the nephridia of a large number of molluscs, quantitatively estimating the uric acid present. It must be emphasised at the outset that if the possibility that a gastropod could excrete uric acid just as fast as it was formed is realised anywhere in practice, the conclusions of this paper will not hold good, although the comparative data for the uric acid retention of molluscan nephridia will still possess a value. The possibility, however, is here taken to be remote. Judgment would be easier if we knew anything detailed about the rapidity of water-flow through the body of molluscs, but as reviews such as that of Adolph [1930] show, very little is known about the water turnover of the body in species other than the frog and some fishes. There is probably not much difference between a marine and a terrestrial environment with respect to the water-flow through the body, for since the osmotic pressure of the molluscan body-fluids is well-known to be approximately equal to that of the sea-water in which they live, no favouring osmotic gradient can be envisaged. Studies of the tonicity of the excretions in molluscs, similar to those of Schwabe [1933] for instance, on the nephridial fluid in crustacea, are urgently needed. On the other hand, fresh-water molluscs, such as *Anodonta*, would be expected to have a much higher water turnover and a copious excretion of hypotonic "urine" from the nephridial orifice. These considerations must be borne in mind in relation to the figures tabulated below.

Arguments in favour of the hypothesis that uricotelic metabolism will reveal itself in high uric acid retention may be drawn from the work of Cuénot [1892; 1894; 1899] on excretion in molluscs studied by means of dyes such as indigo-carmine or safranine. If these dyes, he found, were injected into the body of a gastropod or a lamellibranch mollusc, the concrements in the nephridial cells took them up, and an idea of the rate of excretion could be gained from the time taken to lose dye from the nephridium. "For aquatic molluscs" he writes [1899, p. 77] "one can obtain sufficiently precise figures by injecting a series of animals on one day and then opening them week after week until one observes the decoloration of the kidneys, previously tinted with the injected substance. It seems that in general the nephridial cells accumulate for a very long time within themselves the products which they make, and only excrete them at long intervals; thus in *Anodonta* and *Unio* it was not until 140 days after the injection that the coloured concrements with carminate began to disappear." Similar experiments have been carried out by Emeljanenko [1909] on terrestrial gastropods.

The method adopted in the present research was to dissect out the nephridia[1] from a number of animals (live and in good condition), and after grinding in a mortar with clean sand, to bring the uric acid into solution by digesting the mixed tissue for a shorter or longer time over a small flame with the phosphate mixture of Benedict and Hitchcock [1915] in which uric acid has forty times its normal solubility in water. The final volume represented approximately for each 100 ml. 1 g. wet weight of tissue, but this proportion was often departed from, depending upon the amount of uric acid shown to be present in preliminary trials. Precipitation of the proteins was accomplished by 1·5 % uranium acetate, added in the proportion of about 1 to 10 to the boiling digest, but here also the amount varied according to the quantity of protein present. The uric acid was then estimated on an aliquot part of the final solution, which was always crystal clear and usually colourless, by the colorimetric method of Folin [1933]. This method is known to be unsatisfactory in some cases, but Fujiwara and Kataoka [1933] have carefully surveyed the limits of its specificity. Reduction of phosphotungstic acid under Folin's conditions is brought about by many aromatic amines, phenols, indole derivatives, lactic acid, fructose and inulin. It may be legitimately assumed that none of these compounds would be present in the nephridial tissue to such an extent as to interfere seriously with the conclusion that the main bulk of the colour produced is due to uric acid. The analyses made on the whole body are probably more open to doubt.

In some cases the bodies of the gastropods were not removed fresh from their shells, but the attachment of the columella muscle was previously weakened by placing them for a few seconds (up to half a minute) in boiling water. It was feared that the partial coagulation of the proteins which occurred might have had the effect of rendering the uric acid more difficult to extract with the hot phosphate mixture after grinding, but experiments made in parallel to test this point on several species showed no difference of significance. In many species the small size necessitated the use of the whole body, and it was found that the shells of the organisms formed a satisfactory substitute for the sand used in grinding. In such cases separate determinations of shell weight and live weight on samples of the group permitted the calculation of the wet weight taken.

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[1] For the morphology and cytology of gastropod nephridia see Meisenheimer [1912] and Freitag [1916].

The results are summarised in Table I and placed in order of classification and habitat in Table II. From Table I an idea can be gained of the great variation in size between the different species. The whole body of *Hydrobia* weighs but 3 mg. while the nephridium alone of *Buccinum* weighs well over 1 g., and its whole body would amount to a hundred times as much. Some of the nephridia,

Table I. *Uric acid in molluscs.*

Notation; N, nephridium; WB whole body; RB, rest of body (excluding nephridium); V, visceral mass; M, musculature of body-wall.

Animal	Part used	No. taken	Wet weight each g.	Uric acid	
				mg./g. wet weight	mg./g. dry weight
GASTROPODA					
PULMONATA					
Helix aspersa	WB	3	2·88	1·00	5·43
	WB	3	2·88	0·55	3·00
	WB	33 (young)	0·615	0·48	2·76
	N	20	0·099	29·0	153·0
	N	4	0·09	27·4	167·0
	N	3	0·09	7·8	63·8
	V	3	1·06	0·91	4·43
	V	3	1·65	0·86	4·19
Helicella virgata	WB	40	0·059	1·28	8·05
	WB	25	0·118	3·45	24·8
Helicella itala	WB	9	0·31	0·735	4·63
	WB	6	0·48	0·634	3·98
	WB	6	0·43	0·485	3·05
	N	32	0·0094	31·3	156·0
Cochlicella acuta	WB	56	0·047	3·2	23·0
Bulimulus sporadicus	N	3	0·045	38·3	183·0
Limax maximus	N	1	0·078	30·8	205·0
Limax flavus	N	2	0·019	4·64	31·0
Milax sowerbyi	N	10	0·016	9·9	66·0
Arion ater	N	6	0·064	3·6	36·0
Agriolimax agrestis	N	7	0·036	2·93	20·0
Limnaea stagnalis	N	38	0·030	16·8	137·0
	N	25	0·036	13·4	109·0
	N	29	0·035	11·8	96·5
Planorbis corneus	N	10	0·0145	3·57	19·8
	N	11	0·0106	4·7	26·1
	N	45	0·022	13·3	74·0
	RB	4	0·33	trace	trace
	RB	4	0·32	trace	trace
Limnaea peregra	WB	17	0·048	0·0405	0·245
Ancylastrum fluviatile	WB	93	0·008	0·115	0·58
Onchidella celtica	WB	40	0·206	0·06	0·27
	V	64	0·06	0·098	0·36
	M	64	0·104	0·078	0·49

Table I (*cont.*).

Animal	Part used	No. taken	Wet weight each g.	Uric acid	
				mg./g. wet weight	mg./g. dry weight
OPERCULATA					
Cyclostoma elegans	WB	13	0·154	10·15	64·0
	WB	13	0·213	18·2	114·0
Viviparus fasciatus	N	14	0·018	2·24	17·6
	N	6	0·04	6·6	51·6
	RB	4	0·39	1·02	5·15
	RB	4	0·39	1·09	5·5
Bithynia tentaculata	WB	10	0·079	0·765	5·1
	WB	15	0·083	0·73	4·85
Hydrobia jenkinsi	WB	147	0·0034	nil	nil
	WB	895	0·0021	nil	nil
	WB	450	0·0021	nil	nil
Buccinum undatum	N	2	1·2	0·42	4·17
	N	2	0·67	0·64	6·4
	N	2	1·11	0·42	4·2
	N	1	1·3	0·17	1·7
Gibbula umbilicalis	N	18	0·026	0·23	1·92
	N	9	0·022	0·32	2·65
Monodonta crassa	N	6	0·04	0·56	2·8
	N	2	0·057	0·314	1·57
	N	80	0·025	0·108	0·56
Nassa reticulatus	N	22	0·028	0·47	2·94
Purpura lapillus	N	71	0·02	1·42	5·21
	N	50	0·017	1·92	7·05
	N	51	0·011	0·11	0·88
Turritella communis	N	16	0·0056	1·52	6·0
TECTIBRANCHIATA					
Scaphander lignarius	N	2	0·11	0·212	4·64
NUDIBRANCHIATA					
Doto coronata	WB	8	—	trace	trace
Tritonia hombergi	V	1	4·0	0·96	4·2
	V	3	5·3	0·67	2·9
Coryphella rufibranchiata	WB	1	0·09	trace	trace
Eolis papillosa	WB	2	1·19	0·127	1·16
	WB	9	0·44	0·19	2·1
Onchidorus fusca	WB	1	0·16	trace	trace
Doris tuberculata	V	2	0·43	0·096	0·44
ASPIDOBRANCHIATA (non-operculate)					
Patella vulgata	N	6	0·22	0·124	0·58
	N	13	0·24	0·088	0·41
	V	2	2·33	0·086	0·22
LAMELLIBRANCHIATA					
Anodonta cygnaea	N	16	0·198	nil	nil
Pisidium fontinale	WB	16	0·035	nil	nil
Pecten maximus	N	18	0·169	0·09	0·49
	N	24	0·122	0·099	0·54
Pecten opercularis	N	50	0·028	0·29	2·9
	N	60	0·031	0·49	4·9
AMPHINEURA					
APLACOPHORA					
Proneomenia aglaopheniae	WB	3	—	trace	trace
POLYPLACOPHORA					
Lepidochiton cinereus	WB	21	0·06	0·153	0·77
Acanthochitona crinitus	WB	3	0·19	trace	trace

Table II. *General summary of the observations.*

Animal	Locality	Habitat	mg. uric acid/g. dry weight			
			Nephridium	Whole body	Rest of body	Visc. musc.
PULMONATA						
Pulmonata						
Helix pomatia (Linn.)	France	T	810, 660, 785, 720	—	—	—
Helix aspersa (Müll.)	Cambridge, Plymouth	T	153, 167, 64	5·13, 3·0, 2·76	—	4·43, 4·19
Helicella virgata (da Costa)	Speke's Mill, N. Devon and Marloes, S. Pem.	T	—	8·05, 24·8	—	—
Helicella itala (Linn.)	Marloes, S. Pem. and Wicklow, Ireland	T	156	4·63, 3·98, 3·05	—	—
Cochlicella acuta (Müll.)	Marloes, S. Pem.	T	—	23·0	—	—
Bulimulus sporadicus	Ascension, Paraguay	T	183	—	—	—
Limax maximus (Linn.)	Cambridge	(T)	205	—	—	—
Limax flavus (Linn.)	Cambridge	(T)	31			—
Milax sowerbyi (Fér.)	Cambridge	(T)	66	—	—	—
Arion ater (Linn.)	Cambridge	(T)	36	—	—	—
Agriolimax agrestis (Linn.)	Cambridge	(T)	20	—	—	—
Limnaea stagnalis (Linn.)	Cambridge	FW	137, 109, 96	—	—	—
Planorbis corneus (Linn.)	Cambridge	FW	20, 26, 74	—	trace	—
Limnaea peregra (Müll.)	Speke's Mill, N. Devon	FW	—	0·24	—	—
Ancylastrum (*Pseudancylus*) *fluviatile* (Linn.)	Speke's Mill, N. Devon	FW	—	0·58	—	—
Onchidella celtica (Cuvier)	M.B.L.	SW		0·27	—	0·4, 0·5
Operculata						
Cyclostoma (*Pomatias*) *elegans* (Müll.)	Gt Gaddesden, Herts	T	—	64, 114	—	—
Viviparus (*Paludina*) *fasciatus* (Müll.)	Milton, Cambs.	FW	18, 52	—	5·1 5·5	—
Bithynia tentaculata (Linn.)	Milton, Cambs.	FW	—	5·1, 4·8	—	—
Hydrobia jenkinsi (*Potamopyrgus crystallinus*) (Smith)	Watford and Anglesea	FW	—	0, 0, 0	—	—
Buccinum undatum (Linn.)	M B.L.	SW	4·1, 6·4, 4·2, 1·7	—	—	—
Gibbula umbilicalis (*Trochus umbilicatus*) (da Costa)	M B.L.	SW	1·9, 2·65	—	—	—
Osilinus lineatus (*Monodonta crassa*) (da Costa)	M.B.L. and Hartland	SW	2·8, 1·6, 0·56	—	—	—
Nassarius (*Nassa*) *reticulatus* (Linn.)	M.B.L.	SW	2·9	—	—	—
Nucella (*Purpura*) *lapillus* (Linn.)	M.B.L.	SW	5·2, 7·05, 0·88	—	—	—
Turritella communis (Risso)	M.B.L.	SW	6·0	—	—	—
Littorina littorea (Linn.)	M.B.L. and Hartland	SW	1·3, 1·6, 1·3	trace, 0·2	—	—
Littorina littoralis (*obtusata*) (Linn.)	M.B.L. and Blegberry, N. Devon			0·3, 0·4	—	—
Littorina rudis (*Saxatilis*) (Olivi)	M.B.L. and Blegberry		5·1 —	0·4, 0·5, 1·2, 1·5, 1·5	—	—
Littorina neritoides (Linn.)	M.B.L. and Blegberry	↓ T	—	1·7, 4·3, 0·7, 0·5	—	—
Tectibranchiata						
Scaphander lignarius	M.B.L.	SW	4·64	—	—	—
Nudibranchiata						
Idulia (*Doto*) *coronata* (Gmelin)	M.B.L.	SW	—	trace	—	—
Sphaerostoma (*Tritonia*) *hombergi* (Cuvier)	M.B.L.	SW	—	—	—	4·2, 2·9
Aeolida (*Eolis*) *papillosa* (Linn.)	M.B.L.	SW	—	1·2, 2·1	—	—
Archidoris brittanica (*Doris tuberculata*) (Johnston)	M.B.L.	SW	—	—	—	0·4
Onchidorus fusca	M.B.L.	SW	—	trace	—	—
Coryphella rufibranchiata	M.B.L.	SW	—	trace	—	—
Aspidobranchiata						
Patella vulgata (Linn.)	M.B.L.	SW	0·58, 0·41	—	—	0·22

Table II (*cont.*).

			mg. uric acid/g. dry weight			
Animal	Locality	Habitat	Nephridium	Whole body	Rest of body	Visc. musc.
LAMELLIBRANCHIATA						
Anodonta cygnaea (Linn.)	Elv	FW	0	—	—	—
Pisidium fontinalis (*cinereum*) (Alder)	Milton, Cambs.	FW	—	0	—	—
Pecten maximus (Linn.)	M.B.L.	SW	0·49, 0·51	—	—	—
Aequipecten (*Pecten*) *opercularis* (Linn.)	M.B.L.	SW	2·9, 4·9	—	—	—
AMPHINEURA						
Aplacophora						
Proneomenia aglaopheniae (K.&M.)	M.B.L.	SW	—	trace	—	—
Polyplacophora						
Lepidochiton (*Chiton*) *cinereus* (Linn.)	M.B.L.	SW	—	0·77	—	—
Acanthochitona crinitus (Pennant)	M.B.L.	SW	—	trace	—	—

N.B. The nomenclature adopted in this table follows Ellis [1926] for the terrestrial and fresh-water species, and the Plymouth Fauna List [Marine Biol. Assoc., 1931], as modified by Winckworth [1932; 1934], for the marine ones. Older names are bracketed. Environments are represented by T for terrestrial, (T) for moist or semi-terrestrial, FW for fresh-water, and SW for marine. Under localities M.B.L. stands for English Channel species, collected by the Marine Biological Association at Plymouth. The figures for *Helix pomatia* mostly derive from the previous paper of this series. Correlation of the above results with data on feeding habits may be attempted by consulting the lists of Hirsch [1915], Hunt [1925] and Yonge [1928], but there is no correspondence.

again, are very small; thus those of *Helicella itala* weigh only 9 mg. each, and 20 mg. is an average figure for the nephridia of Trochid marine snails. This involved the taking of large samples, such as nearly a thousand specimens of *Hydrobia* in one case, and from 50 to 100 dissected-out nephridia were a common requirement.

Not until the relation between the results and the environment is brought out, however, do we obtain a coherent picture. In Table II each systematic group is divided into sub-groups according to habitat, terrestrial preceding fresh-water and fresh-water preceding marine.

First of all, the terrestrial pulmonates may be considered. The extremely high retentions of *Helix pomatia* were not encountered in other species, but the uric acid content both of nephridium and whole body is always very high. In *Helix aspersa*[1] the average value for the nephridium would be about 100 and for the whole body 3·5 to 4·0. *H. itala* agrees closely in both ways with *H. aspersa*, but *H. virgata* may contain three times as much in the whole body, as also may *Cochlicella*. All these, as Ellis [1926] says, are typical arid-living forms. At the extreme opposite would be the marine pulmonates, but these are extremely rare, the only convenient English species being *Onchidella celtica*. This very interesting shell-less mollusc (for which see the classical paper of Joyeux-Laffuie [1882]) proved to contain almost no uric acid, its whole body giving a figure of only 0·27 mg./g. dry weight in contradistinction to the amounts from ten to one hundred times as large found in the terrestrial species. It is certain that the high degree of vascularisation of the mantle cavity and the reduction of the gills to vestiges, which characterise the pulmonates, are adaptations to terrestrial life, and *Onchidella* is therefore generally regarded as a return to the sea, *i.e.*

[1] During the dissection of these snails, it was impossible not to be impressed with the large amount of bright yellow pigment excreted by glands in the foot. This was found to be soluble in water but not in chloroform and only irreversibly reducible. Its properties were apparently not those of a flavin or a pterin. The literature is very silent on the subject of gastropod slime-pigments, but there is a reference to them in Krukenberg [1886]. They are now under investigation by my friend Dr Kurt G. Stern.

secondarily aquatic. If, therefore, we may interpret the high retentions seen in the terrestrial pulmonates as evidence of uricotelic metabolism, the condition of affairs in *Onchidella* indicates a far-reaching, if not complete, abandonment of this form of metabolism and a return to ammonia or urea production.

An interesting parallel case to this is to be found in the fresh-water elasmobranchs studied in Siam by Smith and Smith [1931]. The high urea content of elasmobranch blood is now generally believed to be adaptive in character, in that it brings the total osmotic pressure of the internal medium above the external, and so, by forming a favourable osmotic gradient, exempts the fish from the continuous drinking and complicated excretory arrangements found in the teleosts [Smith, *et al.*, 1930, 1; Smith, 1932]. The majority of elasmobranchs are marine, but secondary (or tertiary) penetration into fresh-water environment occurs in certain rather rare cases, such as the saw-fish, *Pristis*. It was accordingly of interest to study the urea content of fresh-water elasmobranch blood, for if no longer required, it might have sunk to a level similar to that of other animals, or on the other hand, if such an evolutionary retrogression were not possible, it might have remained at its usual level[1]. In actuality Smith and Smith observed a diminution of about 60 % in all the species studied. If, however, we were to apply this analogy in full to the case of the marine pulmonate, we should have to suppose that a mixed metabolism existed; true uricotelism supplying only a portion of the total excreted nitrogen, but this we may be reluctant to do in view of the all-or-none character of the cases of uricotelic metabolism hitherto known. It is clear that *Onchidella* invites much closer and more detailed attention.

The remainder of the pulmonates divide into two distinct groups (*a*) the aquatic snails of fresh-water and (*b*) the slugs. The fresh-water pulmonates are all, it is generally agreed, secondary, and correspondingly it is found that the uric acid content of some of them closely approaches that of the terrestrial snails; thus a nephridium may have in *Limnaea* 137 mg./g. dry weight and in *H. aspersa* 153. Nevertheless, there seems to be a gradation here reminiscent of what was seen in the case of *Onchidella*, for *Planorbis* nephridia are not as rich in uric acid as the land-snail nephridia, and the water-snail *L. peregra* with the river limpet *Ancylastrum* have an average body content of only 0·41 mg./g. dry weight, as against 8·75 for the land-snails. This low figure, indeed, closely approaches the 0·27 of *Onchidella*. The apparent conclusion is that some of the fresh-water species are still uricotelic while others are not, and that the difference may be associated with the length of time the species have been in fresh-water. The position of *Ancylastrum* would agree with this view, for as it is a pulmonate it must have entered the fresh waters by an overland route, and yet its completely aquatic limpet-like morphology must indicate a very long stay in fresh-water, long enough for it to diverge so widely from its nearest ally *Planorbis*. However, it is to be remembered that a fresh-water environment probably involves a faster current of water through the body than other environments, and the low values in these species may, therefore, be due to great rapidity of excretion. What has already been said, however, about the excretion of molluscs, makes this possibility only indifferently plausible.

Just as the fresh-water snails vary in uric acid content from "terrestrial" values down to very low ones, so also do the slugs. *L. maximus* gives a nephridial value equal to that of any land-snail, but the others more nearly resemble an intermediate aquatic form such as *Planorbis*. Here again there are two explanations, either that there is a real retrogression from uricotelic metabolism parallel with the loss of the shell, or that the excretory system is more vigorously flushed with water. In favour of the latter view are the recent experiments of Howes and Wells [1934], who also summarise the previous literature on water turnover of terrestrial organisms. Relatively dry and impermeable skins allow of very little loss (*e.g.* mammals 3 %/day, insects 2 %/day, lizard 5 %/day), moist skins allow more (anurans 10 %/day, urodeles 28 %/day). The earthworm may lose 240 %/day in air, but in subterranean conditions, this capacity for loss is probably not a serious deficiency. The slug, however, loses 58 %/day as a minimum in the motionless state, and if continuously creeping, loses 384 %/day. Correspondingly, although snails such as *Helix* can be kept without water for many months, since they only retire into their shells and lose very little, slugs must be supplied with ample water and must constantly drink. As there is clearly so great a current of water through the skin and the slime-glands it is at least very possible that there is also a great current of water through the nephridium and this would have the effect referred to above. On the other hand, the generalisation, which most oecologists would accept, that on the whole slugs inhabit a moister environment than snails, would be in favour of the former alternative. The work on arginase, reported in the succeeding paper of this series by Baldwin [1935], tends also in the same direction.

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[1] The subject of evolutionary retrogression merits more attention from biochemists than it has yet received (*cf.* the papers of Nopcsa [1923] and Blagoveschenski [1929]).

We have now to consider the operculate gastropods. Two groups of them, the terrestrial and the marine, give very clear-cut results. In the first place, *Cyclostoma*, the only English representative of its class, shows an enormous retention of uric acid, exceeding even the figures for *H. pomatia*. Its whole body contains uric acid to the same extent as the nephridium alone of the other terrestrial snails. This finding is no doubt due to the presence in *Cyclostoma* of a "concrementary gland" first described by Claparède [1858] and containing dense deposits of uric acid. A lengthy examination of it was made by Meyer [1925], especially with regard to the symbiotic bacteria which it is said to contain. It is not strictly speaking a gland, but a perivascular agglomeration of large cells containing the concretions. Uric acid was first shown to be present in the gland by Barfurth [1884] and according to Meyer's figures, at least 42 % of its dry weight is uric acid (*cf.* the *H. pomatia* nephridium). Other purine bases are believed to be present also in considerable amounts. Very little is known about the actual excretions of *Cyclostoma*, but the elimination of concrements probably takes place at infrequent intervals, and urea has been recognised by the xanthydrol reaction in the nephridial fluid.

Marine operculate gastropods show, without exception, extremely small amounts of uric acid in nephridium or in whole body. The average figure for all species, 3·2 mg./g. dry weight nephridium, contrasts with the immensely high figures for *Cyclostoma* and the land pulmonates (up to 1000). In other words, it is clear that in these species, of which the whelk *Buccinum* is typical, and which possess no trace of terrestrial ancestry, uricotelic metabolism does not exist. Here the question of water turnover is not likely to affect the issue, since the concentration of the external medium allows of no rapid current through the body.

At this stage the general argument may be crystallised as shown in Table III.

The general conclusion that if uricotelic metabolism can be gauged by uric acid retention, uricotelic metabolism is especially associated with terrestrial life, seems unavoidable. And in this case the comparison is between animals all within a comparatively restricted group, the gastropods.

Table III.

Environment	Typical species		Uric acid, mg./g. dry weight: Nephridium	Uric acid, mg./g. dry weight: Whole body
Terrestrial	Pulm.	*Helix pomatia*	700	(30)
		Helicella itala	150	4
	Operc.	*Cyclostoma elegans*	(1000)	80
Fresh-water	Pulm.	*Limnaea stagnalis*	100	3
	Operc.	*Viviparus fasciatus*	35	1
Marine	Pulm.	*Onchidella celtica*	(0)	0·27
	Operc.	*Buccinum undatum*	4	(0·2)
		Gibbula umbilicalis	2	(0·2)
		Littorina littorea	1	0·2

The figures in brackets are deduced from comparison with other species.

But there remains for consideration the interesting group of fresh-water operculates. It is usually held that these animals, by reason of their gills and lack of mantle vascularisation, must have reached their present position by colonisation up the rivers. We should therefore expect that uricotelic metabolism would not occur in them and that their bodies would be nearly free of uric acid. *Hydrobia jenkinsi* gives a remarkable opportunity of testing this point, for according to Ellis [1926, pp. 31, 78] the species was entirely confined to brackish- and sea-water until near the end of the nineteenth century. Not until 1893 were the first specimens obtained from an inland situation. Subsequently it became abundant in rivers, streams and canals over all the British Isles [see also Boettger, 1931]. In accordance with expectation uric acid was found to be absent from this mollusc, not even detectable amounts being noted in samples from various parts of the country. The phenomenon was sufficiently striking, for where comparable amounts of another gastropod, *Littorina neritoides*, of about the same size (4 mg. individual body weight) would have given deep blue colours, the extract from *H. jenkinsi* was quite colourless. One catch from an Anglesea llynn included some other gastropods; 27 of these (mixed *Limnaea peregra* and *Planorbis albus*) were worked up and gave 1·6 mg./g. dry weight, *i.e.* similar to other fresh-water pulmonates. The difference cannot, therefore, be purely environmental.

On the other hand, *Paludina* and *Bithynia* did not behave as expected, for both of them contained amounts of uric acid corresponding to the fresh-water pulmonates. Recourse to an explanation based on high water turnover is here impossible, for while that would explain a case in which uric acid was expected but not found, it cannot explain a case in which uric acid is found where it was not expected. There seem to be two explanations which are available (1) that uricotelic metabolism is an adaptation not only to terrestrial but also to fresh-water life, (2) that the fresh-water operculates originated not from the sea direct but by way of a period of terrestrial or semi-terrestrial life, analogous to that which *Cyclostoma* and *Littorina neritoides* are now living.

With regard to the first of these possibilities one of the most general characteristics of the fresh-water fauna, as Carter has shown in numerous studies [*e.g.* Carter and Beadle, 1930; 1931] is the possession of adaptations for passing periods of unfavourable conditions inactively. These adaptations are particularly necessary in the fauna of the shallower and often stagnant fresh waters, for the danger of desiccation is always present. Many shallow water animals can pass through periods of drought contracted within their shells. Unfortunately for this point of view, the evidence regarding the capabilities of modern fresh-water operculate gastropods is that they possess very poor powers of withstanding periods of complete dryness. As for the parallel case of the aestivating lung-fishes, it is now definitely known that uricotelic metabolism does not occur in them, but that like the elasmobranchs they can store in their tissues great quantities of urea [Smith *et al.*, 1930, 2]. This mechanism appears to be strictly confined to the fishes.

That the fresh-water operculates originated not directly from the sea but overland in a manner analogous to the fresh-water pulmonates is a suggestion at first sight somewhat more unwelcome to the zoologist, but not by any means impossible on closer inspection. Even in *Cyclostoma* there is a vestigial gill which under suitable conditions might grow again, accompanied by a diminution in the number and size of the blood vessels in the wall of the mantle cavity. And in the periwinkles we see a series of animals the most terrestrial of which is not far removed from the condition of the fresh-water operculates.

The celebrated series of periwinkles affords a particularly good opportunity of testing the ideas here discussed, for within one single genus there is a marked zonation of habitat on the sea-shore, *Littorina littorea* being entirely marine, *L. neritoides* almost wholly terrestrial and two other species, *L. obtusata* and *L. rudis* intermediate. Corresponding with this there is a gradation of adaptations for air-breathing [Carter, 1931]. This is represented in Table II by an arrow, and from the figures accompanying it a gradation in the amount of uric acid contained both in the nephridium and in the whole body, is apparent (Table IV). This is graphically illustrated in Fig. 1. At the bottom of the figure the percentage intertidal exposure (taken from Colman [1932, 1, 2]) is given, showing that whereas *L. littorea* is never exposed for more than 65 % of the time, *L. neritoides* is never exposed for less than 85 % of the time. Moreover, these figures allow for the splash zone. Above, the uric acid in mg./g. dry weight nephridium and whole body is given. Even if the highest value for *L. neritoides* is set aside, there still remains a definite increase with increasing dryness of habitat. At the

Table IV. *The periwinkle series.*

				Uric acid	
Animal	Part used	No. taken	Wet weight each g.	mg./g. wet weight	mg./g. dry weight
Littorina littorea	WB	5	0·48	trace	trace
	WB	20	0·71	0·041	0·205
	N	63	0·027	0·203	1·31
	N	98	0·021	0·255	1·65
	N	26	0·025	0·142	1·31
Littorina obtusata	WB	81	0·082	0·056	0·31
	WB	21	0·08	0·076	0·42
	N	10	0·0085	0·79	5·1
Littorina rudis	WB	66	0·028	0·087	0·41
	WB	154	0·029	0·106	0·50
	WB (large)	42	0·057	—	1·19
	WB (medium)	112	0·026	—	1·54
	WB (small)	232	0·011	—	1·49
Littorina neritoides	WB	140	0·0057	0·338	1·73
	WB	115	0·006	0·851	4·35
	WB	400	0·0063	—	0·71
	WB	755	0·0062	—	0·51

Note. N, nephridium; WB, whole body. Of *L. neritoides* Colman [1932, 1] says it "extends up the rocks several feet above high water mark, where for weeks during a calm summer its sole source of moisture is dew, rain if any, and perhaps the dampness caused by the salt on the rocks." *L. rudis* may also live in positions much above the tide mark: Ellis [1926] lists *L. neritoides* among the terrestrial snails.

point marked *A* is shown the average figure for all the terrestrial pulmonates (excluding their two highest values), and at *B* the value for the single marine pulmonate. It would be tempting to make the preliminary assumption that *L. rudis* and *L. neritoides* are both uricotelic, while the others are not, and this would accord with the fact that the two first-named species are also ovoviviparous. This raises exceedingly interesting general problems which strongly invite much further work on the nitrogen metabolism of the *Littorinidae*. The literature [*cf.* Turchini, 1923] does not seem to contain a comparative cytological study of their nephridia.

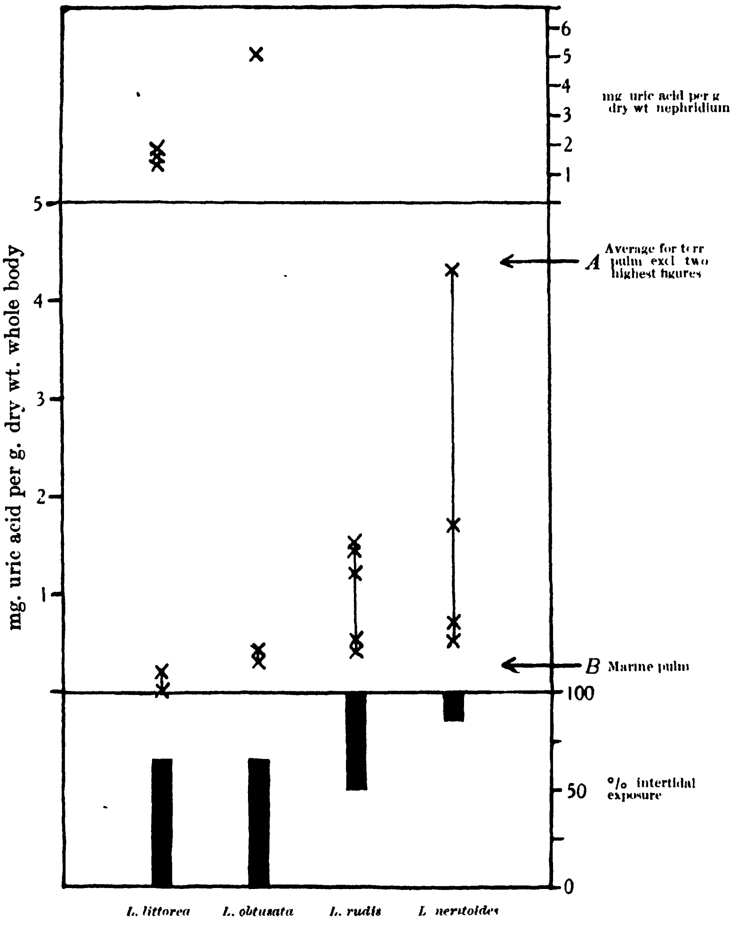

Fig. 1.

The remainder of Table II contains data about various other forms, all marine, belonging to diverse groups such as the Nudibranchs, the Solenogastres *etc.* They are at one in containing little or no uric acid, agreeing therein with their absence of terrestrial ancestry and with the marine gastropods already mentioned. The lamellibranchs were not studied in any detail, but it was curious that while the two fresh-water forms contained no detectable uric acid, the two Pectinidae had measurable amounts. A repeatable difference seems to exist between *P. maximus* and *P. opercularis*, the former having somewhat less uric acid in its nephridium than the latter. The Amphineura gave uniformly negative results.

A few observations were made upon the developing embryos of *Helix aspersa* which may be of interest in view of the absence of any similar information in the literature. The undeveloped eggs contained no uric acid whatever, but the young snails examined immediately after hatching had 2·76 mg./g. dry weight in the whole body. This is analogous to the uric acid formation which Baldwin [1934] found to go on during the development of the pond-snail, *L. stagnalis*. During the development of these *H. aspersa*, which took 23 days, the wet weight of one individual decreased from 37 to 17·5 mg.; a loss of 19·5 mg. or 53 %; while the dry weight decreased from 6·8 to 5·5 mg., *i.e.* by 1·3 mg. or 19 %. The loss of water was therefore 48 %[1]. The uric acid formed per individual was 0·007 mg. or 0·28 % of the final dry weight (excluding the weight of the shell). The comparison of these figures with those for another terrestrial cleidoic uricotelic form, the hen, is interesting. The hen's egg during its development does not lose so much water (about 15 %) probably owing to its more impermeable shell. On the other hand it loses about 18 % of its original dry weight by combustion—a figure quite close to that for the snail. During development, about 35 mg. of uric acid are formed, or 0·7 % of the final dry weight. This is again a figure of the same order as that for the development of the snail. Further work on this material would well repay the effort involved.

It is hoped that the present work may initiate a series of comparative investigations on nitrogen metabolism in molluscs. The lines of advance which most immediately present themselves are (i) the closer examination of nudibranchs, and tectibranchs such as *Aplysia*, *Philine* and *Pleurobranchus* (*Oscanius*), about which conflicting reports exist in the literature, (ii) direct determinations of the nitrogen partition of the excreta in the *Littorina* series, and in selected cases of special interest such as *Hydrobia* and *Onchidella*, (iii) nephridial determinations on the only English fresh-water Aspidobranchiate *Theodoxus* (*Neritina*) so far unobtainable, and on foreign gastropods such as *Ampullaria* which has gill and mantle-cavity of about the same size, (iv) a study of the rapidity of uric acid elimination after injection of the substance or other purines into terrestrial and aquatic species respectively, (v) a thorough examination of the nitrogen metabolism of cephalopods.

SUMMARY.

1. The uric acid retention of the nephridia and other tissues of a large number of molluscs (especially gastropods) has been studied on the assumption that uricotelic metabolism will indicate itself in this way.

2. The reliability of this assumption for molluscs is discussed in the text. If it is correct, the preliminary survey here reported demonstrates that within the homogeneous group of gastropods uricotelic metabolism is an adaptation to terrestrial life.

The author wishes to express his grateful thanks to Mr Hugh Watson, Mr A. E. Ellis, Mr C. Oldham, and Mr F. A. Potts, for their untiring help on matters connected with molluscs, as well in collecting as in identification and theory. He

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[1] In this connection the results of Carmichael and Rivers on the dehydration of slug eggs are of interest. These eggs will still develop even if their wet weight is lowered 85 % by an environment of low humidity.

is also greatly indebted to Dr E. J. Allen for hospitality at the Plymouth laboratory, and to his staff, in particular Mr G. E. Steven and Mr L. H. N. Cooper for their kindness. The expenses of the work were partly defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES.

Adolph (1930). *Quart. Rev. Biol.* **5**, 51.
Baldwin (1934). *J. Exp. Biol.* (in the press).
—— (1935). *Biochem. J.* **29**, 252.
—— and Needham (1934). *Biochem. J.* **28**, 1372.
Barfurth (1884). *Zool. Anz.* **7**, 474.
Benedict and Hitchcock (1915). *J. Biol. Chem.* **20**, 619.
Blagoveschenski (1929). *Biologia Generalis*, **5**, 427.
Boettger (1931). *Arch. Zool. Ital.* **16**, 250.
Carmichael and Rivers (1932). *Ecology*, **13**, 375.
Carter (1931). *Biol. Rev.* **6**, 1.
Carter and Beadle (1930). *J. Linn. Soc.* (*Zool.*), **37**, 205.
—— —— (1931). *J. Linn. Soc.* (*Zool.*), **37**, 327, 380.
Claparède (1858). *Arch. Anat. Physiol. wiss. Med.* 1.
Colman (1932, 1). *J. Mar. Biol. Assoc.* **18**, 435.
—— (1932, 2). *Biol. Bull.* **62**, 3.
Cuénot (1892). *Arch. Biol.* **12**, 683.
—— (1894). *Arch. Zool. Exp. Gen.* (3), **2**, xiii.
—— (1899). *Arch. Biol.* **16**, 77.
Ellis (1926). British snails. (Oxford.)
Emeljanenko (1909). *Z. Biol.* **53**.
Folin (1933). *J. Biol. Chem.* **101**, 111.
Freitag (1916). *Z. wiss. Zool.* **115**, 586.
Fujiwara and Kataoka (1933). *Z. physiol. Chem.* **216**, 133.
Hirsch (1915). *Zool. Jahrb.* (*Zool. u. Physiol.*), **35**, 357.
Hopkins (1928). *Biochem. J.* **22**, 1341.
Howes and Wells (1934). *J. Exp. Biol.* **11**, 327, 344.
Hunt (1925). *J. Mar. Biol. Assoc.* **13**, 560.
Joyeux-Laffuie (1882). *Arch. Zool. Exp. Gen.* **10**, 225.
Krukenberg (1886). Vergleich. Physiol. Vörtr. 145. (Heidelberg.)
Marine Biological Association (1931). Plymouth marine fauna. (Plymouth.)
Meisenheimer (1912). Die Weinbergschnecke. (Leipzig.)
Meyer (1925). *J. Infect. Dis.* **36**, 1.
Needham (1929). *Sci. Prog.* **23**, 623.
—— (1931). Chem. embryol. **2**, 1132. (Cambridge.)
Nopcsa (1923). *Proc. Zool. Soc. Lond.* 1045.
Schwabe (1933). *Z. vergl. Physiol.* **19**, 183.
Simroth and Hoffmann (1928). In Bronn's Klassen u. Ordnungen d. Tierreichs, Mollusca, 3, **2**, 2, p. 413.
Smith (1932). *Quart. Rev. Biol.* **7**, 1.
Smith, Farinacci and Breitweiser (1930, 1). *Amer. J. Physiol.* **93**, 480.
—— —— —— (1930, 2). *J. Biol. Chem.* **88**, 97.
Smith and Smith (1931). *Amer. J. Physiol.* **98**, 279.
Strohl (1914). In Winterstein's Handbuch d. vergl. Physiol. **2**, 443.
Turchini (1923). *Arch. Morph. Gen. Exp. Fasc.* 18.
Winckworth (1932). *J. Conchol.* **19**, 211, 334.
—— 1934. *J. Conchol.* **20**, 9, 51.
Yonge (1928). *Biol. Rev.* **3**, 21.

XXIX. PROBLEMS OF NITROGEN CATABOLISM IN INVERTEBRATES.

III. ARGINASE IN THE INVERTEBRATES, WITH A NEW METHOD FOR ITS DETERMINATION.

By ERNEST BALDWIN[1].

From the Biochemical Department, Cambridge.

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ARGINASE was discovered in mammalian liver by Kossel and Dakin [1904, 1, 2] who showed that the products of its action are ornithine and urea. Since that time the enzyme has attracted a great deal of interest from many points of view, but for present purposes it is sufficient, without going into the history of the subject, to point out that until very recently it was generally supposed that a small proportion of the urea excreted by mammals arises by the action of the liver arginase upon ingested arginine. Krebs and Henseleit, however, brought forward convincing evidence [1932] for supposing that the whole of the urea so excreted is elaborated by means of a cyclical mechanism involving arginase. In the meantime a generalisation of the greatest comparative importance was made by Clementi [1914; 1915], who pointed out that arginase could be detected in the livers of animals having a ureotelic metabolism, but not in cases where the metabolism is uricotelic in character. Manderscheid [1933] has extended the work of Krebs and Henseleit [1932] to other vertebrates and found the same cyclical system operating in those having the ureotelic type of metabolism, thus providing an explanation for the empirical rule enunciated by Clementi.

The distribution of arginase among vertebrate tissues has been carefully studied by a number of workers [Edlbacher, 1915; Hunter and Dauphinee, 1924, 1, 2; Edlbacher and Bonem, 1925] but the invertebrates have received scant attention. Table I presents the results obtained in the few cases hitherto examined. From these results Hunter and Dauphinee [1924, 2] concluded that "arginase is an enzyme almost, if not entirely, peculiar to the vertebrates." But it is known that many and possibly all invertebrates excrete urea to some extent [Delaunay, 1927; 1931]. The facts that *Helix* excretes some 20 % of its waste nitrogen in the form of urea and that it was the only invertebrate then known to contain arginase led Baldwin and Needham [1934] to investigate the possibility of a cyclical synthesis of urea in *Helix* itself. The presence of arginase in

Table I.

Animal and species	Part examined	Arginase	Author
Termite larvae	Whole body	–	Clementi [1918]
Snail (*Helix pomatia*)	Hepatopancreas	+	"
Crayfish (*Astacus fluviatilis*)	"	–	"
Starfish (*Pisaster ochracea*)	Caecae	–	Hunter and Dauphinee [1924, 2]
Crab (*Cancer productus*)	Hepatopancreas	–	
Clam (*Saxidomus giganteus*)	Digestive glands	–	"

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[1] Senior 1851 Student.

the hepatopancreas was confirmed, and the enzyme was also detected in the nephridium, but we were forced to the conclusion that the urea excreted by this snail arises, not synthetically, but from ingested arginine. This brought the snail into the same category as the bird [Clementi, 1932; 1933] and raised the question as to how far the excretion of urea by invertebrates in general can be attributed to the action of a tissue arginase upon ingested arginine. The present paper offers a partial answer to this question in the form of the results obtained for a variety of invertebrates by means of the new and very sensitive method which has been devised for the detection and estimation of arginase.

METHOD.

The essential basis of all the methods which have been employed for the detection of arginase consists in allowing the enzyme to act upon arginine and determining the amount decomposed in one or another of several ways. Of these the most suitable for quantitative purposes is that of estimating the urea which is liberated. The earliest quantitative studies of the distribution of arginase were those of Hunter and Dauphinee [1924, 2] who used a colorimetric method of their own for the estimation of urea [1924, 1]. Their method however was open to objections on theoretical grounds, as was pointed out by Edlbacher and Röthler [1925, 1], who introduced a second quantitative method and defined a new unit of arginase activity; in this case the urea determinations were carried out by a distillation method. But the most delicate method yet introduced for the estimation of urea is that of Krebs and Henseleit [1932]. Here the urea is decomposed by a urease preparation acting at p_H 5; ammonia is effectively bound at this p_H but the CO_2 is liberated and measured manometrically. The sensitivity of this method is at least 100 times that of the distillation method employed by Edlbacher and Röthler [1925, 1], and the manometric technique was therefore adopted for present purposes. It must be mentioned, however, that Weil and Russell [1934] have used the same principle in their studies of blood arginase, their method being published shortly before the present work was completed.

In the method of Edlbacher and Röthler [1925, 1] the arginase was prepared in the form of a glycerol extract of the tissue under examination. Increasing quantities of this extract were incubated for 60 minutes with 10 ml. 1 % arginine solution and 5 ml. of a glycine buffer, the p_H being 9·5 and the temperature 37°. A few drops of toluene were added as an antiseptic. At the end of the period of incubation the enzyme was destroyed by heat, the urea formed being decomposed by urease and the ammonia so set free estimated by titration after being distilled off. A standard curve was now plotted relating urea production to enzyme concentration, the latter being expressed in convenient arbitrary units. The ratio of activity to enzyme concentration was not constant, but fell off with increasing concentration of the enzyme, but the form of the curve was found to be the same for all the tissues examined, with a single exception in the case of the kidney of the bird. This curve could therefore be used as a standard for interpreting the urea production directly in terms of arginase concentration. The number of "Arginase Units" per g. of tissue could then be calculated, and hence the "Arginase Number" of the particular tissue.

This simple procedure however could not be applied at once to invertebrate materials. Different tissues did not, it was found, give superposable curves for the activity-concentration relation, and it was thought that better agreement might be obtained if the enzyme were allowed to act upon an excess of substrate and if the period of its action were reduced. Arginine was therefore used at a concentration of 2 % and an incubation period of 30 minutes only was allowed, and this, since the temperature was 28° instead of 37°, was equivalent to only one-fourth of the period used by Edlbacher and Röthler [1925, 1]. It will be remembered that those authors themselves stressed the importance of measuring the initial rate of the arginase-arginine reaction.

With these modifications it was found that the amount of urea produced was directly proportional to the enzyme concentration, provided that the total urea formed was not more than 4 mg. under the conditions adopted as standard. A number of curves are plotted in Fig. 1 and show this relation clearly. The slope of the straight part of the curve thus gave the urea production per unit weight of tissue, *i.e.* the relative richness of the tissue in arginase. All the results are here expressed in the $Q_{\rm H}$ notation of Krebs and Henseleit [1932], *i.e.* as μl. urea-CO_2 per mg. dry weight of tissue per hour, the temperature being 28°, the activities so expressed being denoted by the symbol $Q_{\rm H}^{28}$. It is possible to express the data given by Edlbacher and Röthler [1925, 2] in the same terms, and it seemed better to use Krebs and Henseleit's mode of expression than to add yet another arbitrary unit to the list already defined.

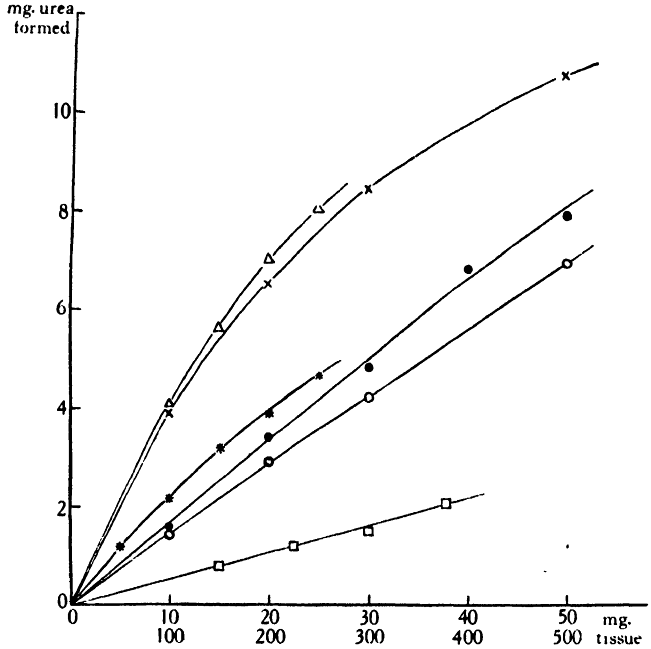

Fig. 1. See text for explanation. * Guinea-pig; • *Limnaea*; o *Viviparus*; × *H. pomatia*; △ *H. aspersa*; □ *Carcinus*. The data for *Carcinus* are plotted on the lower scale of weights.

The conditions of temperature and $p_{\rm H}$ were 28° and 9·5 respectively. Hunter and Dauphinee [1933], Hunter and Morrell [1933] and Hunter [1934] have made a thorough study of the effects of these and other factors upon the reaction and point out that although the enzyme has an optimum $p_{\rm H}$ of 9·5–9·8 under certain conditions [Hunter and Morrell, 1924; Edlbacher and Bonem, 1925] it is very labile at such a $p_{\rm H}$, the lability being much increased by rise of temperature. Consequently there is in practice an optimum neither of temperature nor of p_H but "at p_H 9·8 the optimum for most conditions will be lower than 40° and for many lower than 30°" [Hunter, 1934]. The temperature chosen was 28°; it seemed possible that the enzyme, coming from invertebrate sources, might be more labile than that from vertebrate materials, and this actually appeared to be the case. Fig. 2 shows two comparable curves obtained at p_H 7·4 and 9·5 respectively. That at 7·4 shows that the arginase of *Limnaea stagnalis* is rapidly inactivated even at 28°, whereas mammalian preparations undergo only very slow inactivation under such conditions [Hunter and Dauphinee, 1933]. At 9·5 the *Limnaea* enzyme was practically destroyed in 3 hours, a circumstance which again indicates the desirability of dealing only with the earliest stages of the reaction.

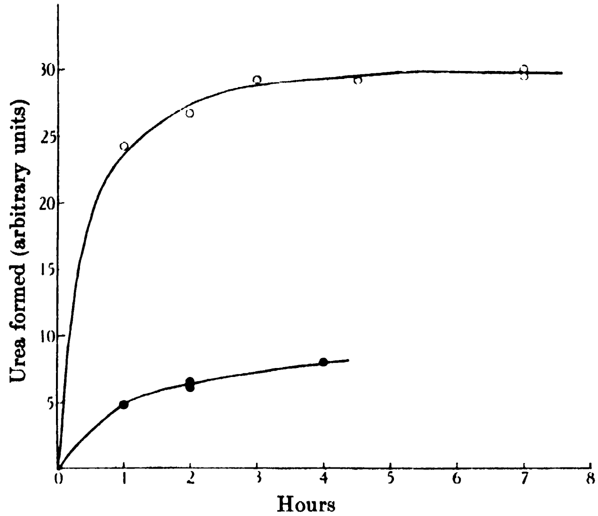

Fig. 2. Time curve of arginase-arginine reaction at 28° (*Limnaea* enzyme). ○ at p_H 9·5; ● at p_H 7·4.

The p_H was controlled by means of a glycine buffer; Weil and Russell [1934] report that such a buffer causes slight inhibition of the arginase of rat blood but no such effect was noticed here. The use of a p_H of 9·5 seems especially suitable since urease, which appears to be of frequent occurrence in invertebrate tissues [Albrecht, 1920; Przyłęcki, 1922; Baldwin and Needham, 1934], is here practically inactive and cannot interfere appreciably with the urea produced by the action of arginase. Indeed, no evidence of such interference was observed during this work.

No antiseptics were employed since the period of incubation was only 30 minutes. Quite apart from any effect it may have had upon arginase itself, the introduction of toluene resulted in vapour pressure effects in the manometers, and stationary readings could not be obtained in spite of prolonged equilibration in the bath. Chloroform was successfully tried in a few of the earlier experiments since, on account of its lower boiling-point, it could be boiled off before samples were taken into the manometric flasks.

Finally, the method of extracting tissue arginase with glycerol has not been employed. At best this is a long and somewhat cumbrous process, and Hunter and Dauphinee [1930] have obtained maximum yields of the enzyme from fresh mammalian liver by extraction with water only for 2 minutes at 60°.

The details of the method may now be described, and it should be pointed out that these details must be closely observed in order to secure comparable results with different tissues.

SOLUTIONS AND EXPERIMENTAL TECHNIQUE.

(1) *Arginine solution.* This contained 5 % of arginine in the form of its carbonate. A little of the Universal Indicator of B.D.H., Ltd. was added and the p_H brought to 9·5 by passing in CO_2.

(2) *Glycine buffer.* A stock solution containing *M* glycine *plus* *M* NaCl was prepared. For use, 7 parts of this received 3 parts of *M* NaOH; such a mixture, diluted 10 times, has a p_H of 9·5 at 28° (interpolated from the data given by Britton [1929]).

(3) *Urease solution and acetate buffer.* These solutions were prepared according to the directions of Krebs and Henseleit [1932], whose method was followed exactly except that for the sake of convenience the estimations were carried out at 28° instead of 37°.

(4) *Preparation of tissue "brei."* The organs selected for analysis were dissected from animals in good physiological condition. Adherent tissues were removed as far as possible, surplus water was absorbed by means of filter-paper, and the material dropped into a chilled dish. When enough had been collected, representative samples were removed and weighed on the torsion balance for the determination of the dry weight, the rest being weighed and transferred to a mortar. Enough sand was added to give a thin paste and the tissue then thoroughly ground. After the addition of a little water the p_H was brought to 9·5 by the addition of *N* NaOH and the mixture filtered through a loose plug of glass wool into a measuring cylinder and washed through. The volume was now made up to the desired value and the p_H finally adjusted if necessary, the Universal Indicator being employed externally.

It is essential that the preparation and neutralisation of the "brei" be carried out as rapidly as possible on account of the lability of arginase.

(5) *Experimental procedure.* A series of pyrex boiling-tubes (6 × 1 in.) were prepared and numbered. Each received 0·5 ml. of the buffer and sufficient water to bring the final volume to 5 ml. A quantity of the arginine solution having been set to warm to 28° in the bath, the "brei" was prepared, and samples were at once measured into the tubes. The latter were then promptly lowered into the bath and left for exactly 5 minutes; 2 ml. of the arginine solution were then added to the contents of each tube (except the control), the additions being made serially and at 10-second intervals. Table II shows the quantities taken in typical experiments. After 30 minutes, the interval being measured with a stop watch, each tube received 1 drop concentrated HCl to neutralise the arginine and break down the buffer, followed by sufficient glacial acetic acid (usually 3–5 drops) to bring about precipitation of the proteins. This brought the p_H to a value at which urease is still fairly active, and the tubes were therefore plunged at once into a briskly boiling water-bath, where they were allowed to remain for

Table II.

Tube		1	2	3	4	5	6 (control)
Buffer	ml.	0·5	0·5	0·5	0·5	0·5	0·5
Water	,,	0·0	0·5	1·0	1·5	2·0	2·0
"Brei"	,,	2·5	2·0	1·5	1·0	0·5	2·5
Arginine	,,	2·0	2·0	2·0	2·0	2·0	0·0

2 minutes. This inactivated the enzymes and usually sufficed to coagulate all the proteins present, which were then filtered off. Crystal clear filtrates, slightly yellow in colour, were usually obtained, and of these samples were taken into the manometric flasks for the determination of their urea contents. In addition to the control on the tissue itself (tube 6 in Table II) additional controls were run on each new lot of the arginine solution, since the arginine used was found to contain a little urea.

(6) *Calculation.* The yields of urea, expressed in terms of μl. of CO_2 and duly corrected for the tissue and solution blanks, were plotted against the amounts of tissue in the corresponding tubes. The yield per mg. of tissue was deduced from the slope of the curve in the manner already indicated, converted into μl. CO_2 per mg. dry weight of tissue and finally expressed as a Q_H^{28} value. It is proposed to refer to each such value as the "arginase index" of the particular tissue.

(7) *Preliminary tests.* Before following out the foregoing somewhat elaborate procedure it was desirable to discover approximately how much arginase, if any at all, was present in each tissue or organ examined. This was done by preparing a rather concentrated "brei," containing 100–300 mg. of tissue per ml. Of this 2·5 ml. were treated in the usual way, the two necessary controls being set up at the same time. Urea estimations were then carried out on the final filtrates, and the arginase index was approximately evaluated; fresh experiments were then set up in all cases to confirm negative results or to secure accurate values for the index where arginase was present. The data in Table II may be regarded as typical of those obtained in such preliminary experiments, but it should be pointed out that arginase was only regarded as present if the total amount of urea CO_2 exceeded 10μl. On the other hand, the disappearance of more than 10μl. CO_2 might conceivably be taken as indicating the presence of urease in the tissue, but such indications would need further confirmation.

Table III.

Animal	*Littorina littorea*	*Mytilus edulis*	*Carcinus maenas* ♂
Part examined	HP	HP	HP
mg. tissue/ml. of "brei"	107	110	94
μl CO_2 found in			
(i) whole	69	79	432
(ii) tissue	0	5	5
(iii) solutions	66	81	66
μl CO_2 formed from arginine	3	-7	361
Arginase	absent	absent	present
Q_H^{28} (approx.)	0·05	0	11

HP, hepatopancreas.

RESULTS AND DISCUSSION.

The results of the experiments performed are presented in Table IV. The liver of the guinea-pig was examined in two cases with a view to comparing the values obtainable for Q_H by the new method and by that of Edlbacher and Röthler [1925, 1, 2]. From the data given by the latter authors it was possible to calculate an index of 930 for female guinea-pigs at 28°, the necessary temperature coefficient being obtained by interpolation from the data given by Hunter [1934][1]. The mean value found by the new method was 750 and it may be taken that the agreement between the two figures is satisfactory in view of the fact that the arginase content of the liver varies considerably from animal to animal as well as with conditions such as age and sex [Edlbacher and Röthler, 1925, 2].

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[1] Krebs and Henseleit [1932] give an index of 4000 for the guinea-pig, but this figure refers to 37° and appears to have been calculated from Edlbacher and Röthler's data for the male animal; furthermore the dry weight was assumed to be 20 % instead of 28 % as was found here.

A glance at the table of results suffices to show that the distribution of arginase is considerably wider than has hitherto been supposed. While two marine lamellibranchs (*Mytilus* and *Pecten*) gave no evidence of containing arginase, thus confirming Hunter and Dauphinee's finding in the case of *Saxidomus*, a fresh water species, *Anodonta*, contained the enzyme. This suggests that the distribution of the enzyme may be somewhat erratic although it is possible that further work would reveal a strict correlation between the presence of arginase and a terrestrial or fresh-water habitat. It will be seen that of all the molluscs examined only the terrestrial and fresh-water species contained the enzyme. The finding of arginase in a crustacean and in an asteroid suggests that it was on account of lack of sensitivity that the enzyme was not detected in *Astacus* and

Table IV.

Phylum and class	Species	Part analysed	No. of specimens	Q_{U}^{28}
CRANIATA	*Cavia* ♀	Liver	1	772
MAMMALIA	(guinea-pig)		1	728
ECHINODERMATA	*Asterias rubens*	Hepatic caecae	1	1·6
ASTEROIDEA	(starfish)		1	1·4
			1 (*a*)	0·9
ARTHROPODA	*Carcinus maenas* ♂	Hepatopancreas	1	*ca.* 11
CRUSTACEA	(shore crab)		1	15·5
			1	19·1
MOLLUSCA	*Mytilus edulis*	,,	3	0·0
LAMELLIBRACHIATA	(mussel)		5	0·0
	Pecten opercularis	,,	3	0·0
	(queen scallop)		5	0·0
	Anodonta cygnaea	,,	4	2·8
	(river mussel)		4	2·9
GASTROPODA	*Buccinum undatum*	,,	1	0·0
	(whelk)		1	0·0
	Littorina littorea	,,	6	0·0
	(periwinkle)		10	0·0
	Patella vulgata	,,	3	0·0
	(limpet)		6	0·0
	Viviparus fasciatus	,,	6	719
	(river snail)			
	Planorbis corneus	,,	12	22·5
	(ram's horn)			
	Limnaea stagnalis	,,	10	682
	(pond snail)	,,	14	587
	Helix aspersa	,,	3	1230
	(common snail)		4	1240
	Helix pomatia	,,	1 (*b*)	1200
	(Roman snail)		2 (*c*)	6500
			2 (*d*)	933
	Arion ater	,,	2	*ca.* 10
	(black slug)		4	11·6
			4	11·6

The nomenclature is that of Ellis [1926].

(*a*) Animal moribund.
(*b*) Starved and aestivating.
(*c*) Just wakened from hibernation and well fed.
(*d*) Just wakened from hibernation but no food eaten.

Pisaster by the methods of Clementi [1918] and Hunter and Dauphinee [1924, 2] respectively. On the other hand the technique of Edlbacher and Röthler [1925, 1] would probably have been sufficiently delicate to allow of the detection even of the small arginase concentrations which were found in the animals so far mentioned, although the indices did not in any case exceed a value of 20, which is less than 3 % of the mean for the guinea-pig livers.

The examination of a moribund specimen of *Asterias* brought to light the fact that arginase content is dependent upon physiological condition, and this was further demonstrated in the case of *Helix pomatia* (see Table IV). Well-fed individuals of this species gave an index of 6500 as compared with 1200 and 933 for starved specimens, while only traces of arginase could be found in the slug *Milax sowerbii* and *Helicella itala* after these had been kept for about a fortnight without food. The results listed in Table IV refer to fresh healthy specimens except in the cases indicated; the marine forms, which were obtained from Plymouth, were in all cases used within a day or two of capture.

Turning now to consider the results obtained for gastropod molluscs, it will be seen that arginase could not be detected in any of the three marine forms which were tested, *viz. Buccinum*, *Littorina* and *Patella.* Yet the hepatopancreases of the fresh-water forms, *Limnaea* and *Viviparus*, contained the enzyme in a concentration quite comparable with that found in mammalian livers. Two terrestrial species, *H. pomatia* and *H. aspersa,* even surpass the mammalia in arginase content, the former having an index of about 6500 and the latter 1200 in well-fed specimens. In a previous paper [Baldwin and Needham, 1934] a value of 20·2 for the hepatopancreas "brei" of *H. pomatia* was published. The experiments in which this value was determined involved incubation of the "brei" with arginine for several hours at p_H 8·4 and 28°. The conclusions of our previous work are not much affected by the newer values here reported, but the fact that such low values were obtained emphasises the importance of measuring only the initial velocity of the arginase-arginine reaction when the arginase index of a given material is to be determined with anything approaching accuracy.

The present finding of indices of from 1000 to 6000 for the hepatopancreases of animals which are primarily uricotelic makes it still more certain that, if there be any homology whatever between the gastropod hepatopancreas and the vertebrate liver, Clementi's rule [1914; 1915] fails in its application to the invertebrata, and all the more so when it is remembered that the nephridium, and perhaps other organs too, contains much arginase [Baldwin and Needham, 1934]. At the same time, in view of the work of Krebs and Henseleit [1932], it seemed somewhat curious that the snail, possessing as it does an arginase content of the same order as that found in mammals, should excrete only 20 % of its waste nitrogen in the form of urea [Delaunay, 1927; 1931], while the mammalia excrete about 80 % in that form.

Meanwhile Needham [1935] was examining the distribution of uric acid in the nephridia of a large number of molluscs, and on comparing our respective results it was found that there appeared to be a correlation between the arginase and uric acid contents of those forms which had been examined in common. Table V shows the average values found for Q_H and for uric acid content, the latter expressed as mg. HU per g. dry weight of nephridium, for those gastropods for which both sets of data are available. A test case of the greatest interest was found in the large black slug, *Arion ater.* As *Arion* appears at first sight to possess all the attributes of a truly terrestrial gastropod, it might have been expected to ally itself with other terrestrial forms such as *Helix* by having a high concentration of nephridial uric acid. This, however, was found not to be

Table V. *Relationship between hepatic arginase and nephridial uric acid in gastropods.*

Species	Habitat	Q_H^{28}	HU content
Buccinum undatum	Marine	0	4·1
Littorina littorea		0	1·5
Patella vulgata		0	0·5
Planorbis corneus	Fresh-water	22·5	40
Limnaea stagnalis		635	114
Viviparus fasciatus		719	35
Helix aspersa	Terrestrial	1235	128
Helix pomatia		6500	744
Arion ater		11	36

Data for Q_H^{28} are the average values from Table IV.
Data for uric acid are mean values calculated from the data of Needham [1935].

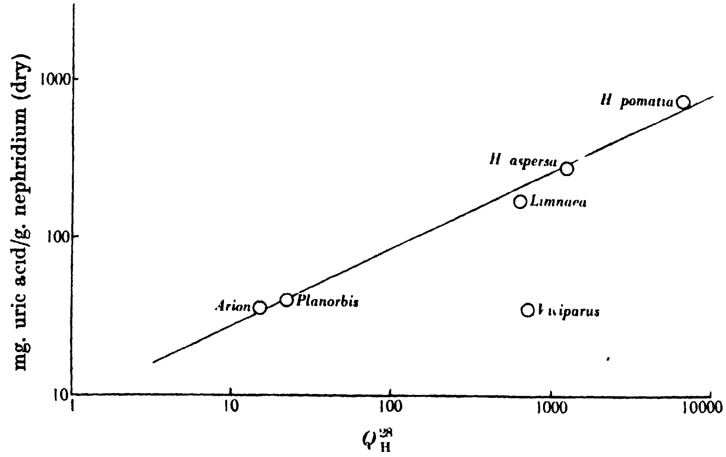

Fig. 3. Relation beween arginase and uric acid in gastropods. (See text.)

the case; the amount of uric acid which could be detected was only a fraction of that to be found in the nephridia of the terrestrial snails as a group, and correlated with this low uric acid content was an arginase index of only 11. This is of the same order as those found for *Carcinus* and *Asterias*, neither of which would be regarded on any grounds as being primarily uricotelic. The relationship between the two sets of data is more clearly shown in Fig. 3, where they have been plotted on a double logarithmic scale. The points for the various forms lie well on the curve, with the exception of that for *Viviparus*, which differs from the others represented in being operculate and not pulmonate and perhaps might therefore be expected to behave in a rather different manner.

We have already seen how Krebs and Henseleit [1932] were able to demonstrate the functional importance of arginase in the elaboration of urea by the mammalian organism, and the discovery of this new and altogether unexpected correlation between arginase content and uric acid formation suggests that the enzyme is also of importance in the elaboration of uric acid by the Gastropoda. This group, together with the Insecta, appears to have specialised more than any other invertebrate group in the production of uric acid as a nitrogenous end-product. To extend this notion to cover the birds, which are the chief producers of uric acid among the vertebrates, would plainly be difficult or impossible on account of the very small amount of arginase which is present in the avian organism [Edlbacher and Röthler, 1925, 2], but this does not detract from the possible value of the new hypothesis, since it has already been shown [Baldwin and Needham, 1934] that the bird and the snail probably make use of quite different chemical mechanisms in the formation of uric acid. Further work is therefore in progress with a view to testing the hypothesis.

A few interesting points remain to be discussed. In the first place, what is the source of the urea which plays such a large part in the nitrogenous excretion of the slug *Arion*? In our examination of the case of *Helix* we were unable to demonstrate any synthetic production of urea and came to the conclusion that the urea excreted by that animal arose almost entirely from ingested arginine under the influence of the arginase of the hepatopancreas and nephridium [Baldwin and Needham, 1934]. It was quite clear that the arginase cycle did not operate in *Helix* in spite of the high arginase index of its hepatopancreas. This must probably be true also in the case of *Arion* whose arginase index is relatively small, but in spite of this *Arion* is believed to excrete a greater proportion of urea than any other invertebrate so far examined [Delaunay, 1927; 1931]. It would be desirable to confirm Delaunay's analyses of the excreta of *Arion*, since, if they are reliable, they must point to the existence of some synthetic mechanism for the production of urea which is markedly different from that found in the vertebrates.

Summary.

1. A new and sensitive method for the detection and estimation of arginase is described, together with a suitable mode of expression of the results.

2. A series of results has been obtained with the aid of this new method, and there is reasonable agreement between the values obtained by the new method and by its predecessor.

3. The examination of a variety of invertebrates has shown that arginase is much more widely distributed than has hitherto been supposed.

4. The arginase content of a given tissue is dependent upon its physiological condition, the enzyme tending to disappear in starvation.

5. On the grounds of a correlation between the arginase and the uric acid content of the hepatopancreases and nephridia respectively in a number of gastropods, it is suggested that arginase is concerned in the production of uric acid by members of this class. Thus in the elaboration both of urea and of uric acid, which represent the principal nitrogenous end-products of the non-sauropsid vertebrates and the gastropods respectively, the same enzyme, arginase, may be involved.

The author is indebted to the Royal Commission for the Exhibition of 1851 for a Senior Studentship during the tenure of which this work was done. He also wishes to thank Dr E. J. Allen for his kind co-operation in providing the marine specimens.

REFERENCES.

Albrecht (1920). *J. Biol. Chem.* **45**, 395.
Baldwin and Needham (1934). *Biochem. J.* **28**, 1372.
Britton (1929). Hydrogen ions. (Chapman and Hall, London.)
Clementi (1914). *Rend. R. Accad. Lincei*, **23**, 612.
—— (1915). *Arch. Fisiol.* **13**, 189.
—— (1918). *Rend. R. Accad. Lincei*, **27**, 299.
—— (1932). *Proc. XIV Congr. Int. Fisiol.* Rome, 24.
—— (1933). *Arch. Sci. Biol.* 18.
Delaunay (1927). *Trav. Sta. Biol. d'Arcachon*, 1.
—— (1931). *Biol. Rev.* **6**, 265.
Edlbacher (1915). *Z. physiol. Chem.* **95**, 81.
—— and Bonem (1925). *Z. physiol. Chem.* **145**, 69.
—— and Röthler (1925, 1). *Z. physiol. Chem.* **148**, 264.
—— —— (1925, 2). *Z. physiol. Chem.* **148**, 273.
Ellis (1926). British snails. (Oxford Univ. Press.)
Hunter (1934). *Quart. J. Exp. Physiol.* **24**, 177.
—— and Dauphinee (1924, 1). *Proc. Roy. Soc. Lond.* B **97**, 209.
—— —— (1924, 2). *Proc. Roy. Soc. Lond.* B **97**, 227.
—— —— (1930). *J. Biol. Chem.* **85**, 627.
—— —— (1933). *Quart. J. Exp. Physiol.* **23**, 119.
—— and Morrell (1924). *J. Soc. Chem. Ind.* **43**, 691.
—— —— (1933). *Quart. J. Exp. Physiol.* **23**, 89.
Kossel and Dakin (1904, 1). *Z. physiol. Chem.* **41**, 321.
—— —— (1904, 2). *Z. physiol. Chem.* **42**, 181.
Krebs and Henseleit (1932). *Z. physiol. Chem.* **210**, 33.
Manderscheid (1933). *Biochem. Z.* **263**, 245.
Needham (1935). *Biochem. J.* **29**, 238.
Przyłęcki (1922). *Arch. Int. Physiol.* **20**, 103.
Weil and Russell (1934). *J. Biol. Chem.* **106**, 505.

XXX. BIOCHEMICAL STUDIES IN THE NITROGEN METABOLISM OF THE APPLE FRUIT.

I. THE ESTIMATION OF AMINO-NITROGEN BY THE VAN SLYKE METHOD IN PRESENCE OF TANNIN.

By ALFRED CRESSWELL HULME.

Low Temperature Research Station, Cambridge, and
Ditton Laboratory, East Malling, Kent.

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Early in a study of changes in the nitrogen fractions of the peel and pulp of the apple fruit during development on the tree serious errors became apparent in the determination of the amino-nitrogen fraction of the total soluble nitrogen by the Van Slyke gasometric method. In certain cases—notably in the peel at all stages of maturity and in the pulp of young fruits—the amino-nitrogen figure obtained was considerably higher than that of the total soluble nitrogen. This fact indicated either that the total soluble nitrogen figures (determined by a modification of the micro-Kjeldahl method to be described in a later communication) were too low or that the "Van Slyke amino-nitrogen" figures were too high. On carrying the investigations further it became apparent that the latter possibility was the more probable. It appeared most likely that some substance present in the extracts, other than amino-acids, was yielding gas in the amino-nitrogen determinations. Various possibilities as to the nature of this substance were examined without success until a paper by Wasteneys and Borsook [1924] was noted in which it was shown that tannic acid, used by these investigators for the precipitation of peptones, yielded large volumes of gas in the Van Slyke apparatus.

Thus it appeared likely that tannin, present in the apple extracts, might be leading to the anomalous amino-nitrogen results [Hulme, 1932].

Support is lent to this view by the results of Overholser and Cruess [1923] who showed that there is considerably more tannin present in the peel than in the pulp of the apple fruit. Thatcher [1915] gives figures varying between 0·132 and 0·208 % of the fresh weight for the tannin content of several varieties of American apples. Wehmer [1929] gives the tannin content of apples as varying between 0·1 and 0·3 % of the fresh weight.

Using the method of Spiers [1914] the present writer found the tannin content of the Bramley's Seedling apples used in this investigation to be of the order of, peel 0·37–0·45 %, pulp 0·06–0·18 % (for criticism of the Spiers method see later). This meant that in the dilutions used (see later) in the Van Slyke amino-nitrogen estimations as much as 50 mg. of tannin might be present. It was therefore decided to investigate the possibility of removing the tannin from extracts prior to the amino-nitrogen determinations and the present paper is concerned with the attainment of this object.

A brief description only will be given of the method of preparing the apple extracts and of other details not directly concerned with the present problem since it is proposed to present such details in full in a later paper in the present series.

EXPERIMENTAL.

1. *General Methods.*

(*a*) *Preparation of the apple extracts.* The method of preparation consisted essentially of extracting the frozen (−20°) and finely ground tissue with 60–80 % alcohol at 40° in a special vacuum-extractor until all the nitrogen soluble under such conditions had been removed. The alcohol was then evaporated off from the extract at 50° under diminished pressure. The pulp extracts had a final volume of 100 ml., contained 100–150 mg. of nitrogen and represented 400 g. of fresh tissue; peel extracts had a final volume of 60 ml., contained 40–100 mg. of nitrogen and represented 100–250 g. of fresh tissue depending on the size and state of maturity of the fruit. The separation of the fruits into peel and pulp tissue was achieved by means of a household potato peeler when the material was in the hard frozen state.

(*b*) *The Van Slyke amino-nitrogen determinations.* The reaction chamber of the apparatus used was intermediate in size between the macro- and micro-forms originally suggested by Van Slyke, the burette used for the introduction of the solution under examination having a maximum capacity of 5 ml. This reaction chamber was used in conjunction with the micro-gas burette (3 ml.). The shaking time for completion of the reaction was 15 min., this time being found necessary for concordant results with apple extracts.

(*c*) *Spiers's method for the estimation of tannin.* It is necessary to outline the essential points of this method as the method of removal of tannin used was adapted to the present problem in preliminary experiments.

Briefly the method consists of titrating a known volume of the tannin-containing solutions (Spiers was chiefly interested in cider), diluted with 750 ml. of distilled water, with potassium permanganate in presence of indigo carmine (as indicator), before and after treatment with fat-free caseinogen. The caseinogen, treatment, which is supposed to remove all the tannin present, consists of shaking 50 ml. of the extract with two successive portions of 1 g. of fat-free caseinogen with intermediate and final filtration through a fine filter medium. The method was standardised by Spiers against Schering's "tannin leviss. puriss." and other commercial tannin preparations.

In the present work Merck's "Caseinum-Natrium for bacteriological purposes" was used after extraction with ether for several hours. Similar results were obtained with a sterile solution of caseinogen prepared from milk by the method of Van Slyke and Baker [1918] kindly supplied by Dr R. B. Haines of the Low Temperature Research Station, who was using such a preparation for bacteriological work. The tannic acid used was Schering-Kahlbaum "acidum tannicum pro analysi" and B.D.H. pure; both these samples gave identical volumes of gas per unit amount in the Van Slyke determinations and their 1 % solutions had approximately the same hydrogen ion concentrations (p_H *ca.* 3·5).

2. *Investigation of the amount of gas given by pure tannic acid solutions in the Van Slyke estimation.*

By "Van Slyke estimation" is meant here and elsewhere throughout the present paper a determination carried out according to the Van Slyke technique for the estimation of amino-nitrogen whether or not the solution under examination contains amino-groups.

Solutions containing various amounts of tannic acid between 5 and 100 mg.

per 5 ml. were prepared and 5 ml. of these solutions were taken for a Van Slyke estimation. Within these limits a linear relation was found to exist between the amount of gas evolved (at 750 mm. and 15°; corrected for blank value) and the amount of tannic acid present, as will be seen from Fig. 1.

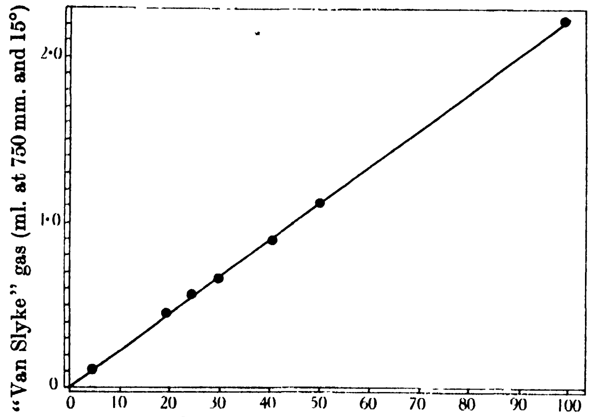

Fig. 1. Tannic acid (mg.)

Thus it is clear that the amounts of tannin likely to be present in apple extracts are sufficient to interfere to an appreciable extent with the amino-nitrogen determinations.

3. *Removal of tannin prior to the Van Slyke estimations.*

(*a*) *Preliminary investigations.* Spiers's method for the estimation of tannin suggests that the treatment of extracts with fat-free caseinogen should bring about a quantitative removal of tannin. If such a method were adopted it would, of course, be essential to remove completely any excess caseinogen before proceeding to an amino-nitrogen determination.

To examine the effect of the addition of caseinogen to pure solutions of tannic acid, two successive portions of 0·5 g. of fat-free caseinogen were added to 50 ml. lots of tannin solutions of various concentrations adjusted to various hydrogen ion concentrations by phosphate or acetate buffers. Filtration through a Whatman No. 44 filter was carried out after each addition of caseinogen and 5 ml. of the filtrates were taken for a Van Slyke determination. The results obtained may be summarised as follows:

(i) The first treatment with caseinogen at p_H 1 to 2 removed (as judged by the reduction in the volume of gas given in the Van Slyke apparatus) about 35 % of the tannin; the second treatment at the same p_H removed all but 19–20 % of the tannin. At this hydrogen ion concentration the caseinogen formed a clot as soon as it entered the tannic acid solution.

(ii) The best results were obtained by two caseinogen treatments at p_H 7 to 8 (which allowed some of the caseinogen to go into solution) followed by the addition of acid to bring the p_H value of the filtrate from the second treatment to 4·6 (to precipitate excess caseinogen), and a further filtration. By this means 96 % of the tannic acid was removed.

When, however, this latter method was applied to apple extracts alone and to extracts to which tannic acid had been added, not more than 40 % of the tannin was removed. Further modifications of the "caseinogen method," including three caseinogen treatments, gave no better results. While this work was in progress a paper by Rahn [1932] appeared in which further emphasis was laid on the serious interference of tannin with the Van Slyke method. Rahn says of tannin "Es reduzierte im Vakuum des Apparates einen Teil des bei der Reaktion entstehenden N_2O_3 zu elementaren N und verhöhte so die vom α-Amino-N stammende N Menge auf das 3–7 fache"—although no evidence was adduced in support of such a theory. Nevertheless Rahn showed that tannin could be removed from plant extracts by precipitation with potassium dichromate. In Rahn's case the tannin had been added for precipitation purposes during the nitrogen fractionation of his extracts; the potassium dichromate solution was allowed to remain in contact with the extracts for a day to bring about the precipitation of the tannin.

Preliminary results for the precipitation of small amounts of tannin from pure tannin solutions by means of dichromate were very promising, so that a more detailed examination of the method was initiated in relation to the present problem. In particular it appeared necessary to ascertain whether the prolonged contact of the dichromate with amino-acids and amides of the asparagine type caused any destruction of these compounds.

(*b*) *The precipitation of tannic acid by dichromate in solutions containing glycine and asparagine.* To 30 ml. of a 1 % solution of tannic acid 3, 4 and 6 ml. quantities of 5 % potassium dichromate were added. The liquids were brought to 50 ml., well shaken and allowed to stand for 18 hours. At the end of this time they were filtered and 5 ml. of the filtrate taken for a Van Slyke estimation. The results obtained are presented in Table I.

Table I.

ml. dichromate	Volume of "Van Slyke" gas (ml. at 750 mm. and 15°)
3	0·23
4	0·17
6	0·18

The solution containing only 3 ml. of dichromate filtered extremely slowly and the filtrate was opalescent. From Fig. 1 it is seen that the volume of gas which would have been given if no tannic acid had been removed is 0·66 ml. (equivalent to 30 mg. tannic acid). Since the water blank value was 0·16 it is clear that 4 and 6 ml. of dichromate solution were sufficient to remove all the tannic acid present.

To examine the effect of dichromate on glycine and asparagine and to ascertain whether the dichromate effected a quantitative removal of tannin in the presence of these compounds and also if such a removal were affected by the p_H of the solution, a series of experiments was carried out along the following lines.

To 5 ml. of a solution containing 0·5 g. of pure glycine and 0·5 g. of pure asparagine per 100 ml. of solution was added phosphate or acetate buffer to give the required p_H. Five ml. of a 5 % solution of tannic acid were added together with 2–4 ml. of 5 % potassium dichromate solution and distilled water to make the volume to 45 ml. The mixture was well shaken and allowed to stand for 18 to 20 hours at 1° (to minimise any effect of the dichromate on the glycine and asparagine). At the end of this time the liquid was filtered through a fine filter and 5 ml. of the filtrate were taken for a Van Slyke estimation.

Similar mixtures were prepared with distilled water in place of the tannic acid and also in the absence of dichromate. The results obtained are given in Table II. The amounts of glycine, asparagine and tannic acid present and the ml. gas obtained in the Van Slyke determination all refer to the 5 ml. of filtrate used in the

Van Slyke estimation. The dichromate volume refers to that initially present in the whole solution. The gas volumes are all corrected to 750 mm. and 15°.

Table II.

	Glycine mg.	Asparagine mg.	Tannic acid mg.	Dichromate ml.	ml. gas (theoretical)	ml. gas (observed)	p_{H}
(1)	2·767	—	—	4	0·94	1·14	4·6
(2)	2·767	2·767	—	4	1·45	1·61	3·8
(3)	2·767	—	28	—	1·58	1·66	3·9
(4)	2·767	—	28	4	0·94	1·00	3·2
(5)	2·767	2·767	—	—	2·09	2·06	3·9
(6)	2·767	2·767	28	—	1·45	1·42	3·4
(7)	2·767	2·767	28	4	As (6) assuming all	1·41	3·2
(8)	2·767	2·767	28	4	tannic acid to be	1·48	3·4
(9)	2·767	2·767	28	4	precipitated	1·44	5·0
(10)	2·767	2·767	28	4	„	1·18	5·5
(11)	2·767	2·767	6	2	„	1·16	5·7
(12)	2·767	2·767	28	4	„	1·19	6·4
(13)	2·767	2·767	28	4	„	1·10	6·8
(14)	2·767	2·767	28	4	„	1·20	7·5

Results (1) and (2) give the "blank" value (av. 0·18 ml.) which is applied to all the subsequent results. The "theoretical" values are calculated from the amino-nitrogen content of the glycine and asparagine present, assuming glycine to give 103 % of its calculated nitrogen content in the Van Slyke apparatus [Mitchell and Hamilton, 1929]; the "theoretical" value for tannic acid is obtained from Fig. 1.

p_{H} values were measured by means of a Morton glass electrode. Filtration was extremely slow in the case of mixtures (12), (13) and (14) and the filtrates were turbid.

From Table II it appears that all the tannin is removed from solution by the dichromate treatment without destruction or adsorption of glycine or asparagine between p_{H} 3·2 and 5·0. Above this p_{H} range the low values for amino-nitrogen were probably due to some adsorption of the glycine and/or asparagine on the tannin-dichromate precipitate. Between p_{H} 3·2 and 5·0 the precipitates of tannin-dichromate complex were reddish brown and granular, changing to dark brown and gelatinous with increase of the p_{H}.

(*c*) *The tannin-dichromate precipitation in presence of apple fruit extracts.* The effect of dichromate solution on the amino-nitrogen figure of apple extracts (pulp and peel) alone and in the presence of added tannic acid, and on the amino-figure for apple extracts *plus* added glycine-asparagine solutions with and without added tannic acid was next investigated.

A brief description of the method of preparation of the apple extracts has already been given. 10 ml. of each extract were nearly neutralised with NaOH and 5 ml. of a 10 % suspension of magnesia added. The free ammonia was then distilled off under diminished pressure at 40° in a modification of the apparatus employed by Chibnall and Westall [1932]. The excess magnesia in the residue was dissolved in a minimum of warm glacial acetic acid and the liquid transferred to a 50 ml. standard flask. The requisite amount of 5 % dichromate solution was then added, followed by distilled water to the 50 ml. mark. Where glycine-asparagine solution (0·5 g. glycine and 0·5 g. asparagine per 100 ml.) and tannic acid solution (5 %) were added, this was done and the requisite buffer added before the addition of the dichromate. After bringing to the 50 ml. mark the flask was shaken vigorously for a few minutes and then placed at 1° for the times stated. The tannin precipitation is a "time reaction." The solution first gradually darkens and then the precipitate slowly forms, the whole process taking 10–30 seconds. The precipitate, under the most favourable conditions of hydrogen ion concentration, does not settle for several hours. Shaking greatly accelerates the process.

5 ml. of the filtrate from the tannin precipitation were taken for the Van Slyke estimations. The results obtained are given in Table III. As before, the figures for glycine, asparagine and ml. gas (corrected) refer to the 5 ml. of solution actually taken for the Van Slyke determination, and the volume of dichromate solution refers to that present in the 50 ml. of liquid before filtration.

Table III.

Material	p_H	ml. gas* (expected)	ml. gas (observed)
Stood for 18 hours before filtration:			
Pulp (E. 1)			
Extract alone	4·6	—	1·31
,, +4 ml. dichromate	4·1	—	0·83
,, +1·5 mg. glycine +1·5 mg. asparagine +4 ml. dichromate	4·1	1·60	1·60
,, +1·5 mg. glycine +1·5 mg. asparagine +25 mg. tannic acid +4 ml. dichromate	4·0	1·60	1·57
Stood for 42 hours:			
Pulp (E. 3)			
Extract alone	3·7	—	1·82
,, +2 ml. dichromate	4·0	—	1·11
,, +1·5 mg. glycine +1·5 mg. asparagine +2 ml. dichromate	4·0	1·88	1·90
,, +1·5 mg. glycine +1·5 mg. asparagine +2 ml. dichromate	3·6	1·88	1·91
,, +1·5 mg. glycine +1·5 mg. asparagine +4 ml. dichromate	3·9	1·88	1·84
Stood for 18–20 hours:			
Peel (E. 6)			
Extract alone	4·4	—	2·39
,, +4 ml. dichromate	3·8	—	0·89
,, +2 mg. glycine +2 mg. asparagine +4 ml. dichromate	3·7	1·92	1·95
Pulp (E. 5)			
Extract alone	3·9	—	1·68
,, +2 ml. dichromate	3·9	—	1·30
,, +4 ml. dichromate	3·8	—	1·29

* Assuming all the tannic acid to be removed by "dichromate-precipitation."

From the table it is clear that the dichromate treatment removes added tannic acid from apple extracts without destruction or removal of added glycine and asparagine. Presumably also the dichromate removes tannin from the apple extracts themselves. The fact that a much larger proportional decrease in the amount of gas given in the Van Slyke estimation follows the dichromate treatment in the case of peel than of pulp extracts provides corroborative evidence for such a suggestion, since, as has already been shown, the peel of apples contains much more tannin than does the pulp. The hydrion concentration of the medium does not appear to affect the precipitation of tannin at least between p_H 3·6 and 4·4. The precipitates in the case of apple extracts were very similar in colour and texture to those obtained with pure tannic acid-glycine–asparagine solutions. Even when 25 mg. of tannic acid were present in addition to that of the extracts it is seen that 4 ml. of 5 % potassium dichromate per 50 ml. of solution were sufficient to remove all tannin. It appears to be important not to use a large excess of dichromate, for 5 ml. of undiluted 5 % dichromate solution gave a "Van Slyke blank" value which was about double the water blank. In the above results the blank value obtained from results (1) and (2) in Table II was applied. This is the only satisfactory blank obtainable since the amount of free dichromate after tannin precipitation in each individual case is unknown. This blank value is only 0·02 ml. greater than the water blank and, as the maximum variation in repeated estimations is 0·04 ml., this difference is negligible.

Here may be given a typical result illustrating the relative effects on the Van Slyke figure for an apple extract of the caseinogen and the dichromate treatments. In Table IV are given the observed amounts of "amino-N" in 100 ml. of an extract of a sample of young apples (after removal of free ammonia).

Table IV.

	Direct	After caseinogen treatment	After dichromate treatment
mg. "amino-N"	39·0	35·0	26·2

This and similar results readily explain the high Van Slyke amino-nitrogen figures mentioned in the introduction.

Finally it was necessary to ascertain whether there was any adsorption of nitrogen (amino-groups) on the dichromate precipitates in the case of apple extracts. With this object in view the residues from the estimation of free ammonia in 10 ml. of extract, after neutralisation of the excess magnesia with acetic acid, were washed into a 50 ml. flask, dichromate solution was added and the liquid brought to 50 ml. After standing for 18-20 hours at 1° the precipitate formed was filtered off. 5 ml. aliquot parts of the filtrate were taken for a Van Slyke estimation. Other 5 ml. portions were evaporated nearly to dryness on a water-bath with 2 ml. of concentrated sulphuric acid. The nitrogen contents of these residues were then determined by a modification of the micro-Kjeldahl method using hydrogen peroxide as catalyst. These were compared with the total nitrogen content of an equivalent amount of original extract, after deducting the free ammonia-nitrogen. The results are given in Table V.

Table V.

		Total N	ml. gas in Van Slyke
Pulp (V).	Extract alone	1·53	2·10
	,, +4 ml. dichromate	1·49	1·40
Pulp (IVc).	Extract alone	2·17	3·20
	,, +2 ml. dichromate	2·06	1·96
	,, +3 ml. dichromate	2·13	1·93

These results show that while the amino-nitrogen figure may be reduced by the dichromate treatment by as much as 40 % the reduction is due to the removal of a non-nitrogenous substance since, under the same conditions, the total nitrogen is reduced by only about 2 %.

The final method adopted for the estimation of amino-nitrogen in apple pulp and peel extracts is as follows.

The excess magnesia present in the residue from a free ammonia estimation on 10 ml. of extract is dissolved in a minimum of glacial acetic acid and the mixture transferred to a 50 ml. standard flask. 3 ml. of 5% potassium dichromate solution are then added, and the liquid is brought to the 50 ml. mark with distilled water. After shaking well the flask is allowed to stand overnight at 1°. The dichromate-tannin precipitate is then filtered off, using a small Whatman No. 44 paper, and 5 ml. of the filtrate are taken for the usual Van Slyke determination. No p_H adjustment is necessary prior to the addition of dichromate as it was found that the p_H of the "neutralised" and diluted ammonia residues was always in the region of p_H 3·8 to 4·4.

No doubt the method is applicable to other tannin-containing plant extracts.

The fact that such small amounts of tannin give appreciable amounts of gas in the Van Slyke estimation casts serious doubts on many of the results hitherto reported for the amino-acid content of plant material. Also the data presented make it necessary to question the accuracy of Spiers's method for the estimation of tannin (depending as it does on the quantitative removal of tannin by precipitation with caseinogen), especially when applied to complex solutions such as cider and plant extracts generally.

Strange as it may appear on first consideration, it may be possible to make use of the Van Slyke amino-nitrogen technique in the estimation of the tannin content of plant material. For, since the amount of gas given by tannin in the Van Slyke estimation is a linear function of the amount of tannin present, and since dichromate appears to precipitate tannin quantitatively without affecting the true amino-nitrogen figure, the reduction in the "Van Slyke gas" volume brought about by the treatment with dichromate should be a quantitative measure of the amount of tannin present. However a method based on these facts would depend entirely on a calibration with oak gall tannin (the "purest" standard at present available commercially), and it may be argued, since the chemistry of the tannins is still somewhat obscure, that tannin from other sources may not give the same volume of gas per unit amount in the Van Slyke apparatus. Oak gall tannin is a mixture of pyrogallol tannins, whereas, for example, apple tannin appears to belong to the group of catechol tannins [Overholser and Cruess, 1923]. Nevertheless a similar objection applies to the method of Spiers and to other methods except that relative (generally to oak gall tannin) permanganate titration, relative weight of strychnine precipitate *etc.*, are used instead of the proposed Van Slyke gas volume. It is proposed to investigate such a method as outlined above for the determination of tannin in apples. Carried out in conjunction with amino-acid determination it should prove convenient, rapid and valuable, at least as a means of determining the relative tannin contents of a series of plant extracts.

One final suggestion which arises from the present results. It may be that potassium dichromate could be used with advantage to replace lead salts and other precipitants for plant extracts in certain cases.

SUMMARY.

1. Results are given which suggest that the abnormally high figures obtained for the amino-nitrogen content of apple fruits by the Van Slyke method are due to the presence of tannin.

2. It is shown that a linear relation exists between the amount of gas given in the Van Slyke estimation in the case of solutions of pure commercial preparations of tannic acid (from oak galls). As little as 10 mg. of tannic acid gives 0·20 ml. of gas in the "Van Slyke."

3. Attempts to remove tannin from pure tannin solutions and from apple extracts by precipitation with caseinogen are described. While suitable conditions can be found for thus achieving the removal of 96 % of the tannin from the

former, not more than 40 % can be removed from apple extracts by this means.

4. Experiments are described which show that complete removal of small amounts of tannic acid added to apple extracts can be achieved by precipitation with potassium dichromate. Presumably, also, tannic acid present in the extracts themselves is removed by this method which is shown not to interfere with amino- or acid amide groups.

5. A method, based on this result, is described whereby the interference of small amounts of tannin with the Van Slyke amino-nitrogen determination is entirely overcome.

6. Tentative suggestions are advanced for a new method for determining the tannin content of plant extracts.

REFERENCES.

Chibnall and Westall (1932). *Biochem. J.* **26**, 122.
Hulme (1932). *Report of the Food Investigation Board*, p. 78.
Mitchell and Hamilton (1929). The biochemistry of the amino-acids. (New York.)
Overholser and Cruess (1923). *Agric. Exp. Sta. Univ. Calif. Agric. Coll. Tech. Paper* No. 7.
Rahn (1932). *Planta*, **18**, 1.
Spiers (1914). *J. Agric. Sci.* **6**, 77.
Thatcher (1915). *J. Agric. Res.* **5**, 103.
Van Slyke and Baker (1918). *J. Biol. Chem.* **35**, 127.
Wasteneys and Borsook (1924). *J. Biol. Chem.* **62**, 1.
Wehmer (1929). Die Pflanzenstoffe. (Zweite Auflage, Jena.)

XXXI. IODINE IN CABBAGE.

By JESSE FRANCIS McCLENDON and
CURTIS ETHAN HOLDRIDGE.

From the Laboratory of Physiological Chemistry, University of Minnesota Medical School.

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In iodine analyses of foodstuffs the most difficult part of the technique is the destruction of organic matter without the loss of iodine, and therefore only that part of the method will be mentioned, the remainder being given by McClendon [1934, pp. 304–309].

The cabbages were sliced and dried in a current of air at slightly above room temperature, then ground in a mill, and dried in an oven at 100°. Weighed samples were packed in Visking sausage casing and burned in a modified combustion tube (see Fig. 1). The results of the analyses are shown in Table I. In order to compare them with previous data we have computed the average values of samples from two sections of the State. It will be seen that those from one section are higher in iodine than those from the other.

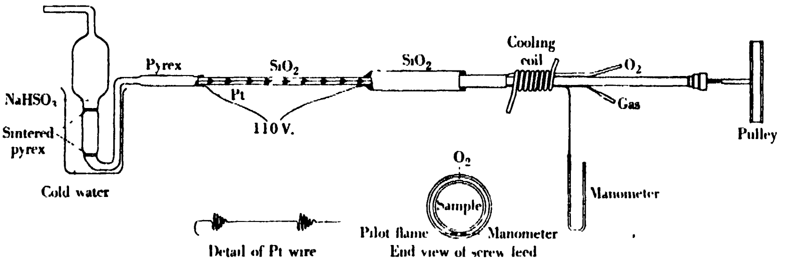

Fig. 1. Apparatus for burning dried tissue for iodine analysis. A detail of the opening of the screw-feed is shown below. The central circle is the space containing the dried tissue packed in a Visking sausage casing and round it is an annular opening from which oxygen issues to burn the tissue (one small circle below is an opening for a gas pilot flame and the other is for the manometer, used to ensure a zero or slightly negative pressure in the combustion tube). The screw is advanced by means of a belt connecting the pulley with the worm reducing gear of an electric motor.

Minnesota may be divided into two portions in relation to goitre and iodine. The most goitrous region and the one containing the least amount of iodine is the Arrowhead region (north-east), which is the region with the least soil on the top of the granite (the soil having been rubbed off by glaciers) and also the region of the greatest rainfall. The region of the most iodine and least goitre is the western part of the State. During the Permian period this region was near the shores of a gulf connected with the Pacific Ocean through Kansas, New Mexico and Arizona, but was cut off later by the rise of the Rocky Mountains. In the intermediate region one of these merges into the other and the division between them is rather indefinite so that for our purposes the line drawn between them has been made rather arbitrarily. In the table the iodine analyses of cabbage

Table I.

West and South Minnesota Counties.

County	Iodine γ/kg. dry Cabbage	Iodine γ/kg. dry Potato	Goitre per 1000 draft
Kittson	—	—	17
Roseau	210	—	8
L. of the Woods	—	63	—
Marshall	—	—	8
Pennington	186	—	5
Red Lake	—	—	—
Polk	65	344	10
Beltrami	—	—	4
Hubbard	—	—	6
Becker	160	—	8
Norman	—	—	3
Clay	81	—	—
Wilkin	—	—	13
Ottertail	—	—	6
Wadena	—	179	12
Todd	—	—	4
Douglas	—	—	5
Traverse	—	—	5
Stevens	511	225	—
Pope	—	—	5
Stearns	—	—	7
Meeker	—	—	5
Kandiyohi	152	329	3
Lac qui Parle	—	—	17
Chippewa	—	—	19
Yellow Med.	—	—	2
McLeod	187	135	—
Sibley	—	—	12
Nicollet	—	—	7
Brown	—	—	1
Redwood	—	—	31
Lincoln	—	—	4
Pipe Stone	84	38	5
Murray	258	—	—
Cottonwood	139	—	—
Watonwan	—	—	4
Blue Earth	—	—	2
Waseca	73	565	4
Rock	—	—	12
Martin	164	164	8
Freeborn	114	—	5
Mower	—	—	5
Fillmore	—	—	12
Houston	225	—	27
Average	174	227	8·5

North-east and East Minnesota Counties.

County	Iodine γ/kg. dry Cabbage	Iodine γ/kg. dry Potato	Goitre per 1000 draft
Cook	16	32	24
Lake	—	123	31
St Louis	53	117	24
Koochiching	155	172	3
Itasca	—	36	—
Cass	127	—	5
Crow Wing	—	—	—
Aitkin	142	—	7
Carlton	81	—	39
Pine	238	118	19
Kanabec	—	—	—
Mille Lacs	53	127	—
Benton	83	—	8
Sherburne	—	—	10
Isanti	—	—	—
Chisago	—	—	22
Anoka	—	75	4
Washington	—	—	38
Ramsey	123	—	3
Hennepin	100	—	11
Wright	—	—	10
Carver	—	—	9
Scott	—	—	—
Dakota	88	—	22
Rice	—	—	5
Goodhue	100	—	15
Wabasha	192	—	37
Winona	—	106	30
Dodge	—	—	19
Average	111	101	17·2

are compared with those by McClendon *et al.* [1934] on potato, together with the goitre per thousand drafted men during the Great War. No data are available for Clearwater, Grant, Swift, Big Stone, Renville, Lyon, Nobles, Jackson, Faribault or Olmsted counties in the south-west section or for Isanti or Scott in the east-north-east section. In the east-north-east there were cabbages from 14 counties, potatoes from 9, and Draft Board data on goitre from 37. In the east-north-east section the average value of iodine in cabbage was 111 γ/kg. dry material, in potato 101, and goitre 17·2 per thousand men. In the west-south, iodine in γ/kg. cabbage (dry) averaged 174, in potato 227, and goitre per thousand drafted men 8·5 (the War Department defined goitre as a neck too large to button a military collar round).

These data indicate that goitre is associated with iodine deficiency in the foodstuffs eaten as well as in the water drunk, as shown by previous analyses. McClendon and Hathaway found 0·01 γ/kg. in the only drinking water not contaminated with sewage in the east-north-east section that was examined, whereas in the west-south section the 4 samples analysed contained 0·05, 0·05, 0·08[1] and 0·14 γ/kg. The work on water was undertaken on the assumption that the iodine content of drinking water was related in some way to the iodine content of water soaked up by plants and evaporated from the leaves and hence to the iodine content of the plants. This water is evaporated from the leaves of plants and hence iodine is concentrated in the leaves. The potato was first chosen because it was easy to obtain, but later cabbage was chosen because it consists of leaves. From the samples available the outer leaves, open to free evaporation, had been removed, and hence the iodine content was lower than it would be in such expanded leaves in which the iodide sucked up by the roots with the soil water is concentrated by evaporation. Owing to the fact that cabbage was the only leafy vegetable obtainable from these counties we have had to content ourselves with analyses of food of relatively low iodine content.

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[1] River with sewage from 5000 population, whereas water omitted from east-north-east study contained sewage from about 100,000 population.

REFERENCES.

McClendon (1934). Manual of biochemistry. (Wiley, New York.)
—— Barrett and Canniff (1934). *Biochem. J.* **28**, 1209.

XXXII. OBSERVATIONS ON THE CHEMICAL METHOD FOR THE ESTIMATION OF VITAMIN C.

By BASHIR AHMAD.

From the Department of Biochemistry and Nutrition, All-India Institute of Hygiene and Public Health, Calcutta.

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Since Svirbely and Szent-Györgyi [1932] showed experimentally that vitamin C was identical with hexuronic or ascorbic acid, the reducing property of the latter has been utilised for the estimation of vitamin C by a chemical method. Tillmans and his colleagues using the indicator 2 : 6-dichlorophenolindophenol estimated the reducing value of certain foods and correlated it with their vitamin C content. Following the same technique, Birch *et al.* [1933] have developed a simple method for the vitamin C assay of natural foodstuffs. The method consists in preparing a trichloroacetic acid extract of the material and rapidly titrating it with 2 : 6-dichlorophenolindophenol. These authors have put forward substantial evidence to show that under the conditions of the test naturally occurring substances other than ascorbic acid, which are likely to reduce the indicator, do not interfere with the estimation, and the values so obtained for a large number of fruits and vegetables agree with their antiscorbutic values as estimated by the biological method.

This simple method has obvious advantages over the long and tedious biological technique. More recent work, however, appears to indicate the existence of unknown reducing substances which may interfere with the test [Svirbely, 1933; Harris, 1933]. At the same time ascorbic acid is known to undergo a reversible oxidative change which does not detract from its value as an antiscorbutic, but in that state ascorbic acid will not react with the indicator dichlorophenolindophenol. Recently Guha and Ghosh [1934] have pointed out that trichloroacetic acid by itself would reduce the indicator even in a considerably dilute solution. Further, trichloroacetic acid can by no means be expected to extract quantitatively the ascorbic acid present in the material, and the proportion extracted may vary from material to material depending on the state in which it is held within the tissues.

These observations appear to show that the chemical method may be liable to errors of a rather disturbing magnitude. Opportunity was afforded to study this method during examination of the nutritional values of Indian foodstuffs which is being undertaken in this laboratory. The results of these studies are reported in this paper.

Experimental.

(1) *The reaction of trichloroacetic and ascorbic acids with the indicator*[1].

We have confirmed the observation of Guha and Ghosh [1934] that trichloroacetic acid reduces the indicator (Table I). The reaction takes place slowly, more

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[1] The indicator solution referred to in this paper was of the strength

1 ml. indicator = 1 mg. ascorbic acid.

It was standardised against pure ascorbic acid solution which was in turn standardised with 0·01 N iodine solution.

Table I. *Reaction of trichloroacetic acid with 2 : 6-dichlorophenolindophenol.*

Concentration of trichloroacetic acid %	Volume of trichloroacetic acid added to 0·1 ml. of indicator ml.	Time of complete reduction mins.
50·0	0·5	0·75– 1·0
20·0	0·5	0·75– 1·0
10·0	0·5	1·0 – 1·25
5·0	0·5	2·0 – 2·25
1·0	0·5	13·0
1·0	1·0	8·0
1·0	5·0	6·0
0·5	0·5	20·0
0·5	1·0	15·0
0·5	5·0	10·0
0·1	1·0	40·0
0·1	5·0	30·0
0·1	10·0	25·0

so in lower concentrations when the time taken to reduce 0·1 ml. of the indicator is considerably prolonged. On the other hand the reaction of ascorbic acid solution with the indicator is quicker, being in fact almost instantaneous (Table II).

Table II. *Reaction of ascorbic acid with 2 : 6-dichlorophenolindophenol.*

Concentration of ascorbic acid mg./100 ml.	Volume of indicator solution used ml.	Volume of ascorbic acid solution required to reduce the indicator ml.	Time for completion of reaction mins.
100·0	1·00	1·05	Instantaneous
50·0	1·00	2·10	„
10·0	0·50	5·25	„
5·0	0·50	10·50	0·75–1·0
1·0	0·10	10·50	2·0 –2·5
0·5	0·10	21·00	3·0
0·1	0·02	21·00	5·0

When, however, a mixture of trichloroacetic and ascorbic acids is titrated against the indicator, the reaction depends upon three factors: (i) the concentration of trichloroacetic acid, (ii) the concentration of ascorbic acid, and (iii) the time taken to complete the titration.

When the concentration of ascorbic acid is not below a certain limit and the concentration of trichloroacetic acid is not very high, and the titration is finished reasonably quickly, the estimation of ascorbic acid is not interfered with by the presence of trichloroacetic acid. If, however, the concentration of ascorbic acid is low, the interaction of trichloroacetic acid with the indicator introduces a significant error in the estimation. These observations will be clear from a study of Table III.

These results show that when the concentration of ascorbic acid in the solution is over 2 mg./100 ml., even 10 % of trichloroacetic acid will not interfere with the titration of ascorbic acid with the indicator. Birch *et al.* [1933] have recommended a concentration of 5 % trichloroacetic acid in the vegetable extracts at the time of titration. Therefore it seems that this concentration is safe even for slightly higher dilutions than 2 mg./100 ml. of ascorbic acid.

Table III. *Reaction of a mixture of trichloroacetic and ascorbic acids with the indicator.*

Concentration of trichloroacetic acid %	Concentration of ascorbic acid mg./100 ml.	Actual volume of mixture required to reduce 0·2 ml. of indicator ml.	Volume predicted from the concentration of ascorbic acid ml.
0·0	10·80	1·90	1·85
10·0	5·40	3·70	3·70
10·0	2·16	9·25	9·25
10·0	1·08	14·00	18·50
5·0	5·40	3·70	3·70
5·0	2·16	9·25	9·25
5·0	1·08	18·30	18·50
2·5	5·40	3·70	3·70
2·5	2·16	9·25	9·25
2·5	1·08	18·50	18·50

When ascorbic acid solutions are allowed to stand in the laboratory under ordinary conditions, even after a short time their titration value with the indicator falls, apparently owing to oxidation. But when the solutions are acidified with a drop of trichloroacetic or even acetic acid at the time of titration the value is found to be higher than when titrated without the addition of the acid (Table IV).

Table IV. *Reaction of the partly oxidised solutions of ascorbic acid with the indicator.*

Time the solution was allowed to stand	Volume of solution required to reduce 0·2 ml. indicator ml.	Volume of solution required to reduce 0·2 ml. indicator after adding 1 drop of glacial acetic acid ml.
0	1·85	1·85
10 mins.	1·90	1·85
30 „	2·10	1·85
3 hrs.	2·20	1·85
24 „	2·40	2·00
72 „	3·60	3·25

It appears from these observations (Table IV) that even 10 minutes after the preparation of ascorbic acid solution the titration value is lowered, showing an appreciable oxidation of ascorbic acid. The oxidation continues and at the end of 3 hours the apparent loss is about 16 %. Up to this time one drop of glacial acetic acid added to the titration flask at the time of titration would bring the reading back to normal. As more time passes the reading does not come back to normal on acidifying though it always gives a higher value for ascorbic acid than when titrated in the unacidified form. It appears that a part of the oxidised ascorbic acid, and presumably the reversibly oxidised form, returns to the reduced state on the addition of the acid.

(2) *The titration of natural fruit juices with the indicator.*

For extracts of natural fruit juices the results of the estimation of ascorbic acid are less likely to be vitiated by the presence of trichloroacetic acid than those for pure solutions. Different dilutions of orange and lemon juices were titrated with the indicator in the presence of 5 % trichloroacetic acid. The readings were theoretically correct when the titrations were finished within 1–2 minutes. On the other hand, when the titration was delayed to 3–5 minutes, high values for ascorbic acid were obtained (Tables V and VI). Lemon juice mixed with known quantities of ascorbic acid solution also gave theoretical values.

Table V. *The titration of orange juice with the indicator.*

Concentration of juice %	Concentration of ascorbic acid in juice mg./100 ml.	Concentration of trichloroacetic acid %	For 0·5 ml. indicator	
			Predicted reading	Actual reading
100	31·25	0	—	1·60
25	7·81	5	6·4	6·4
20	6·25	5	8·0	8·0
25*	7·81	5	6·4	6·2
25†	7·81	5	6·4	6·0

* Titration delayed to 3 minutes.
† Titration delayed to 5 minutes.
In all other cases the titration was finished within 1–2 minutes.

Table VI. *The titration of lemon juice with the indicator.*

Concentration of juice %	Concentration of ascorbic acid in the juice mg./100 ml.	Concentration of trichloroacetic acid %	For 0·5 ml. indicator	
			Predicted reading	Actual reading
100	25·8	0	—	0·425
100	25·8	5	0·425	0·425
10	2·58	5	4·25	4·25
4	1·03	5	10·65	10·65
2	0·515	5	21·5	21·5

(3) *The extraction of ascorbic acid from fruits and vegetables by trichloroacetic acid.*

Birch *et al.* [1933] have recommended the use of trichloroacetic acid for the extraction of vitamin C from natural food materials. Trichloroacetic acid may precipitate protein matter and help to break up the cell-walls, thus facilitating the extraction of ascorbic acid, and may stabilise the vitamin so extracted by inhibiting the action of oxidising enzymes. But it can not be stated what proportion of the vitamin actually present in the tissues comes out in the extract. To investigate this question properly it would be necessary to test the various fractions of extracts and residues by animal feeding experiments. We, however, attempted to obtain maximum yields of ascorbic acid by modifying the method and time of extraction, varying the concentration of trichloroacetic acid used, repeating the extractions, and by the application of heat *etc.* The results of these experiments are summarised in Tables VII and VIII.

It appears from the results summarised in Table VII that 20–25 % is the optimum concentration of trichloroacetic acid to extract the maximum quantity of ascorbic acid. Boiling the vegetable with water or trichloroacetic acid did not increase the amount of vitamin C in the extract. But leaving the shredded vegetable exposed to the warm atmosphere may destroy over 67 % of the vitamin during the course of 2 hours. Storing the vegetable as such also has a very deleterious effect.

The results shown in Table VII refer to a single extraction. By repeating the extractions more reducing substance is obtained as shown in Table VIII.

Table VII.

10 g. of karela[1] were ground up with sand and trichloroacetic acid and filtered. The extract together with washings was made up to 25 ml. The final concentration of trichloroacetic acid in the diluted extracts was maintained at 5 %.

Concentration of trichloroacetic acid used for extraction	Volume required for 0·25 ml. of indicator ml.	Ascorbic acid value as calculated from the reading mg./100 g.
100	3·35	18·65
75	3·25	19·23
50	2·85	21·93
25*	1·80	34·72
20*	1·80	34·72
10†	3·0	20·83
5	3·5	17·86

* Boiling the vegetable for 5–10 minutes with water or trichloroacetic acid and then making the extracts had no effect upon the titration values.

† Cutting up or shredding the vegetable and leaving it for some time before making the extracts profoundly affected the values, *e.g.*

	Reading	Ascorbic acid value mg./100 g.
Fresh	1·20	52·08
After 10 minutes	1·35	46·30
After 20 minutes	1·45	43·10
After 2 hours	3·50	17·85
Keeping whole vegetable for 3 days at room temperature	6·50	10·00

Table VIII.

Vegetable and treatment	Reading for 0·1 ml. indicator ml.	Ascorbic acid value mg./100 g.
10 g. karela extracted in cold with 20 % trichloroacetic acid, extract made up to 25 ml. Concentration of trichloroacetic acid in the extract 5 %.		
1. First extraction of vegetable	0·50	50·0
2. Extraction of the residue	1·90	13·1
3. Further extraction of the residue	20·0	1·25
4. ,, ,, ,,	50·0	0·50
10 g. cabbage treated in the same manner as karela.		
1. First extraction of vegetable	2·5	11·0
2. Extraction of residue	25·0	1·0
3. Further extraction of the residue	40·0	0·62
4. ,, ,, ,,	—	0

This simple experiment shows that a single extraction with trichloroacetic acid does not extract anything like the full amount of vitamin C present in the vegetable tissues. Supposing the four repeated extractions to represent the full amount of vitamin C, then the first extraction represents only 77·1 % in the case of karela and 87·1 % in the case of cabbage.

(4) *The effect of boiling or cooking on cabbage.*

Cabbage behaved differently from most other vegetables in our experience. Usually boiling the vegetable with water or trichloroacetic acid did not profoundly affect the ascorbic acid value of the extract. But boiling the cabbage for 10 minutes raised the ascorbic acid value almost threefold (Table IX). Boiling for a longer time resulted in the destruction of the reducing substance in the extract.

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[1] Karela (*Momordica charantia*, Curcurbitaceae) is a common Indian vegetable, selected on account of its richness in vitamin C.

Table IX.

10 g. cabbage extracted with 6·25 ml. of 20 % trichloroacetic acid either in the cold or with boiling. Extracts made up to 25 ml.

Treatment	Ascorbic acid value mg./100 g.
Cold extraction	13·3
Boiling 10 minutes	40·5
,, 15 ,,	29·15
,, 30 ,,	22·30
,, 1 hour	14·8
,, 1½ ,,	5·0
,, 2 ,,	1·56

It may appear from these observations that either the cell walls of cabbage are not broken up by grinding with cold trichloroacetic acid or the vitamin is more firmly bound within the tissues.

CONCLUSIONS.

The experiments described in the text of this paper show that this method can be of practical value for the estimation of ascorbic acid in its solutions and in natural materials. Under definite conditions it is capable of giving accurate and quantitative results. Trichloroacetic acid which slowly reduces the indicator by itself does not interfere in the estimation if the titrations are carried out quickly and finished within 1–2 minutes. On the other hand, in the presence of trichloroacetic acid and by titrating the solutions against the indicator instead of the reverse procedure of Tillmans, a better and sharper end-point is obtained. Solutions of pure ascorbic acid, natural fruit juices and mixtures of both in varying dilutions show theoretically correct results under the following conditions:

(i) The concentration of ascorbic acid in solutions or extracts in which estimation is desired must be higher than 2 mg./100 ml.

(ii) The concentration of trichloroacetic acid must not be higher than 5 %.

(iii) 1·0–0·1 ml. of 0·01 *M* solutions of the indicator are to be placed in the titration flask according to the potency of the extract, and the solution is to be run in from the burette steadily with constant shaking of the flask.

(iv) The titration is to be finished within 1–2 minutes.

Difficulty arises when the solutions to be titrated are weak in ascorbic acid content, containing less than 2 or even 1 mg./100 ml. Vegetables and fruits poor in vitamin C often yield extracts of this quality. In these cases the reaction is slow, the end-point indefinite, and consequently the values are only roughly approximate.

The next question which needs consideration is whether reversibly oxidised ascorbic acid, which is probably as good an antiscorbutic as the reduced form, can be estimated by the chemical method. Ordinarily the indicator will not react with this form of ascorbic acid and, when the latter constitutes an appreciable proportion of the whole, the results may be quite misleading. Some authors have utilised the action of H_2S over a prolonged period to bring the acid back to the reduced form [Eekelen *et al.*, 1933]. But it appears undesirable to treat a complex natural material with H_2S for such a long period as 6 hours, on account of the unknown changes that the treatment may induce. Further, it is not known whether this reversibly oxidised ascorbic acid exists in appreciable amounts in natural vegetable tissues.

The experiments summarised in Table IV show that a certain proportion of ascorbic acid which ordinarily does not react with the indicator does so in the presence of trichloroacetic or glacial acetic acid.

With respect to the extraction of vitamin C from fruits and vegetables, 20–25 % trichloroacetic acid appears to give the best results. Higher concentrations probably destroy the vitamin and lower concentrations do not extract it completely. Boiling the vegetable tissues with water or trichloroacetic acid solutions does not materially raise the quantity of ascorbic acid in the extracts excepting in that of cabbage. On repeated extraction of the residue, however, more of the vitamin is extracted. In the two vegetables investigated the first extract represented only 77–87 % of the total ascorbic acid. This throws considerable doubt on the value of this method of extraction, as only a proportion of the total vitamin is extracted, and this proportion may vary from material to material. It is clear that this method does not give quantitatively the vitamin present in the vegetable material. At the same time we cannot be sure that in the biological estimation of vitamin C the whole of ascorbic acid present in the food is absorbed and taken account of by the organism. Probably the efficiencies of the two processes run more or less parallel, and hence the striking agreement between the values obtained by the two methods.

Attention may also be drawn here to the ready destruction of vitamin C in vegetable tissues when left exposed to the atmosphere after being cut or shredded. In karela over 67 % of the vitamin was destroyed during 2 hours. A similar observation has been recorded by Kohman *et al.* [1931] for carrots. Storing karelas for three days at room temperature reduced their vitamin C by over 80 %. These points are of practical importance from the point of view of nutrition, for they show how considerably vegetables may vary in their vitamin C content according to their freshness.

I wish to express my deep indebtedness to Prof. H. E. C. Wilson for his constant interest, help and criticism throughout the course of this investigation, and to the Director, All-India Institute of Hygiene and Public Health, for permission to publish these results.

REFERENCES.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Eekelen, Emmerie, Josephy and Wolff (1933). *Nature*, **132**, 315.
Guha and Gosh (1934). *Current Science*, **2**, 390.
Harris (1933). *Nature*, **132**, 605.
Kohman, Eddy and Gurin (1931). *Ind. Eng. Chem.* **23**, 808.
Svirbely (1933). *Biochem. J.* **27**, 960.
—— and Szent-Györgyi (1932). *Biochem. J.* **26**, 865.

XXXIII. THE VARIATION IN THE METHOXYL AND CELLULOSE VALUES OF THE FIBRE OF THE FRUIT OF *COCOS NUCIFERA* DURING ITS GROWTH FROM INFANCY OF THE NUT TO ITS ULTIMATE DRYING ON THE TREE.

BY SREEKANDATH RAMAN KUTTY MENON.

From the Presidency College, Madras.

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THE belief is held by coir workers on the Malabar Coast that nuts about ten months old yield the best fibre (in colour as well as in strength), and accordingly all the high quality coir from Allepey and Cochin is the product of nuts of this age.

The subject is one that has not received adequate scientific investigation [*cf.* Barker, 1933]. The present work records the determination of "Cross and Bevan cellulose" and of methoxyl in samples of fibre from nuts of different ages, the latter being regarded as an index of the extent of lignification.

EXPERIMENTAL.

Preparation of samples for analysis.

Coconuts of different ages from the youngest to the ripest on the trees were collected. It was difficult to persuade coir workers to extract the fibre from unretted husks of young nuts.

The husk was first of all peeled to pieces, soaked in water for a day and then heated to boiling in water with a little tamarind, the latter being supposed to hasten dissolution of the gummy matter and liberation of the fibre from the corky tissues. After this the husks were beaten out, and the bulk of the corky refuse was removed by repeated shuffling and combing.

The crude fibre samples obtained in this way were brought to Madras and further treated as follows. After boiling for 10 minutes with 20 % acetic acid[1], the samples were alternately beaten with a pestle on an inverted mortar and washed vigorously under the tap for an hour, followed by removal by hand of the last traces of corky matter. Finally they were washed with distilled water and air-dried.

Grinding of the samples for analysis presented difficulty and the device was adopted of cropping a bundle of fibre with a "Kodak trimmer," followed by sieving through a 40-mesh sieve. The powdered samples were preserved in stoppered weighing bottles.

Physical appearance of the samples.

The physical appearance and quality of the fibres varied considerably. That extracted from the infant nut was pale yellow in colour, shrivelled, exceedingly thin and much shorter than the fibres of the older nuts. The colour grew more intense and the thickness of the fibre increased with age until the best colour with maximum gloss was found in the fibre of the nut that was 10 months old. Thereafter the fibre reddened and became "drier" with increasing age of the nut.

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[1] The use of dilute acetic acid is thought not to affect the chemical nature of the fibre. Thus Cross and Bevan [1903] showed that in the case of jute the action of dilute mineral acids consisted only in a physical dissolution of the fibre, and the dissolved portion isolated from the solution resembled the original fibre in all chemical properties.

Analyses.

Moisture was determined by vacuum-drying at 110°. The percentages ranged from 7 to 13, 10 % being a usual figure. Other determinations were made on air-dried samples, the results being then calculated on a dry basis. Methoxyl determinations were carried out by Zeisel's method.

Estimation of "Cross and Bevan cellulose."

Coir fibre proved unexpectedly resistant to chlorination when the original Cross and Bevan method [1889] was employed, this resistance increasing with the age of the nut to a maximum at the 10 months stage and decreasing thereafter. The following modification of the method was found to give consistent results.

A small sample (less than 0·5 g.) is boiled for half an hour (in a 100 ml. beaker with a glass rod) with 40 ml. of 1 % caustic soda solution, allowed to settle and washed thoroughly with hot water by decantation, the first liquor and washings being poured through a weighed sintered glass crucible. The moist fibre is then spread over the sides of the beaker, and the latter with the crucible exposed to a slow stream of chlorine in a glass chamber with gas inlet and outlet. The fibre is well washed by decantation as before and then heated slowly to boiling with 40 ml. of 2 % sodium sulphite solution; 0·1 % of sodium hydroxide (calculated on the weight of the solution) is then added and boiling continued for 5 minutes.

After again washing by decantation as much as possible the fibre is transferred by means of a glass rod and the aid of a jet from a wash-bottle, both from the beaker and the crucible to a small glass mortar. After trituration the fibre is again chlorinated (in the mortar). This treatment is found to lessen the number of chlorinations required. The whole series of operations is repeated until a uniformly yellowish-white cellulose is obtained, as many as five chlorinations being necessary in the case of the mature fibre, although three are enough for the infant coconut fibre.

The crude cellulose is finally purified by bleaching with dilute permanganate solution followed by sulphurous acid in the usual manner, washed on the filter, acidified with acetic acid, again washed and dried at 110° to constant weight. The crucible is weighed in a weighing bottle since the cellulose is somewhat hygroscopic.

By this procedure results concordant within 3 % could be obtained. The following table shows the results obtained:

Approximate age of the nut from which fibre was extracted	Mean value of the methoxyl percentage	Mean value of the "Cross and Bevan cellulose" percentage
3 months	3·9	66
5 ,, (tender coconut)	4·3	62
7 months	5·0	59
10 ,, (mature nut, but husk green)	5·7	53
12 months	5·3	57

The figures on the dry nut were not consistent and so are not included; the methoxyl percentage of this sample was however below and the cellulose percentage above the corresponding values for fibre from the 10 month nut.

DISCUSSION.

The results show a maximum for the methoxyl value and a minimum for the cellulose value for the fibre from nuts 10 months old, that is the nuts most favoured on the Malabar coast for the manufacture of coir.

The possibility is borne in mind that the tannin matter which increases in quantity as the nut matures, may be a disintegration product of lignin and that the apparent increase in percentage of cellulose after a minimum has been reached may be due to elimination of the lignin component. Further work on this and allied subjects is projected.

I have to express my thanks to Prof. B. B. Dey, for providing facilities for the present work.

REFERENCES.

Barker (1933). Empire Marketing Board Rep. No. 71.
Cross and Bevan (1889). *J. Chem. Soc.* **55**, 199.
—— —— (1903). Cellulose. (London.)

XXXIV. HORMONES IN THE URINE FOLLOWING OOPHORECTOMY DURING PREGNANCY.

By HAMISH ALLAN and EDWARD CHARLES DODDS.

From the Courtauld Institute of Biochemistry, Middlesex Hospital.

(*Received December 18th, 1934.*)

The operation of bilateral oophorectomy during pregnancy is not commonly performed, yet there is a large number of cases on record. In the majority, the pregnancy was carried to term without interruption. Very few cases, however, have been reported in which any attempt has been made to study the hormones present in blood and urine after operation. So far as we are aware, only four such cases have been studied; these are reported by Amati [1928], Waldstein [1929], Szarka [1930] and Saidl [1932]. Table I gives a summary of their results.

Table I.

				Hormones post-operation			
				A.L.H.		Oestrone	
Authority	Age of patient	Clinical condition	Hormones pre-operation	Blood	Urine	Blood	Urine
Amati [1928]	?	Dermoid cysts	—	—	—	Present	—
Waldstein [1929]	26	Dermoid cysts	—	0·6 ml. Serum positive	1·8 ml. Positive	1–2 ml. Positive	400–500 M.U. per litre
Szarka [1930]	33	Dermoid cysts	—	—	—	5 ml. 4 units	1 ml. Positive
Saidl [1932]	23	Dermoid cysts	A.L.H. 900 M.U. per litre Ov.H. 500 M.U. per litre	—	650–1250 M.U. per litre	Positive in 1·8 ml.	450–680 M.U. per litre

* Weaker solution not tried.

Through the kindness of Mr Aleck Bourne it was made possible for us, during the autumn of 1932, to study a fifth case of this type. In this instance bilateral oophorectomy was performed about three months after conception.

Unfortunately, after the patient's discharge from hospital she returned to her home, some fifty miles from London, and a further move about three months later brought the experiment to an end. The period during which specimens have been obtainable is about 108 days, beginning 112 days after the last menstruation and ending about 50 days before delivery.

Owing to the available stocks of experimental animals the estimations of hypophyseal hormone were carried out first, and it is these only on which a report is now made. A small number of oestrone estimations were carried out as described below.

Clinical details.

Mrs K. M. Age 36, Nullipara. Admitted to hospital on account of abdominal pain and swelling.

Operation 27. ix. 32 by Mr Aleck Bourne—Bilateral oophorectomy. Pathologically these were found to consist of multiple lutein cysts. Convalescence was uneventful and patient returned home on 17th day Delivery at ten months of a normal, healthy child. The child was not breast-fed.

Examination of urine and blood.

A. *Pre-operative.*

(1) *Urine.* Since information of the approaching operation was only received a few hours before it actually occurred only a small quantity of urine was available for test. There was sufficient for a rough oestrone test (7 rats only), giving a value of 2000 M.U. per litre. Tested for hypophyseal hormone, a positive result was obtained with 0·18 ml. urine.

(2) *Blood.* No blood was obtained for test before operation.

B. *Post-operative.*

The tests for hypophyseal hormone were carried out using batches of five infantile mice, 21 days old on first day of test. Each animal received 6 doses of 0·3 ml. of urine (or diluted urine) during 48 hours. They were killed after 96 hours and the ovaries examined with a hand lens. The total number of corpora lutea or haemorrhagica in the group of five mice has been recorded in the table, together with total weight in mg. of 8 ovaries and 8 uterine horns.

A few further tests, not included in Table II, were carried out, in the hope of obtaining a better qualitative result by the use of larger numbers of mice. The last such test, 112 days after operation gave, out of 48 mice, 12 showing positive results, *i.e.* presence of corpora lutea or haemorrhagica.

Table II. *Response to injections.*

(Indicated by 1 unit for every 10 (or fraction of 10) C.L. or C.H.)

Days post-operation	Total wt. of mice g.	Wt. of genitalia g.	Undiluted urine	Diluted 1 in 5	Diluted 1 in 10	Diluted 1 in 20	Minimum effective vol. urine	
0	—	—	6	4	1	0	0·06	Oestrone test. 2000 M.U. per litre
1	(*a*) 37·8	0·175	9	5	4	0	0·06	
	(*b*) 39·5	—	—	—	—	—	—	
				1 in 7·5	1 in 15			Oestrone test. 1400 M.U. per litre
2	50·1	2·240	10	3	3	2	0·03	
*6	45·5	0·133	4	1	0	0	0·12	
9	45·6	0·233	3	0	0	0	0·60	
14	39·0	0·230	4	1	0	0	0·08	
16	43·6	0·262	5	1	0	0	0·08	
	49·3	0·221	3	1	0	0	0·08	
21	54·0	0·260	4	—	—	—	—	
				1 in 5	1 in 10	—	—	Blood. Hypophyseal hormone positive (0·03 ml. serum)
27	45·4	0·262	4	—	—	—	—	
	44·0	0·298	3	0	0	—	—	
29	43·5	0·137	7	1	1	—	0·06	
	48·0	0·209	6	0	0	—	0·6	
*33	48·5	0·187	4	—	—	—	—	
35	49·0	0·264	3	0	0	—	0·6	
	46·5	0·166	5	—	—	—	—	
40	40·5	0·280	2	0	0	—	0·6	
47	(*a*) 90·2	0·141	6	1	0	—	0·3	
	(*b*) —	0·199	4	—	—	—	—	
54	41·6	0·243	4	1	0	—	0·3	Oestrone test. 1300 M.U. per litre
*62	46·5	0·129	5	1	0	—	0·3	
69	44·6	0·194	4	1	0	—	0·3	
73	46·2	0·202	4	1	0	—	0·3	
			Dilution 1 in 2·5					
80	—	—	3	—	—	—	—	

* Indicates approximate time of menstrual period in absence of pregnancy.

Examination of Table II brings to light that there is no significant change in the excretion of hypophyseal hormone brought about by the operation, apart from a very brief rise on the first and second days after operation.

Comparison of present results with earlier workers.

The experiments of Amati were not quantitative and his results have since been confirmed and amplified. Waldstein's figure for oestrone, while agreeing with that of Saidl, is lower than that obtained in the present case. In this connection it must be pointed out that no details of oestrone assay are given by Saidl, and that Waldstein appears to have used the method of diminishing doses—*i.e.* in a batch of animals each one receives a different dose, and the smallest dose to give a positive reaction is taken as containing 1 unit. This method is now known to give unreliable results. In regard to our own figure, *i.e.* 1400 M.U. per litre, it must be admitted that a relatively small number of animals (30) was employed, but it is probably more accurate than an estimation by the method of diminishing doses. The figures for anterior lobe hormone (A.L.H.) given by Saidl show that he obtained a steady increase from 150 to 1250 M.U. per litre in the course of 10 days after operation.

In the present case a similar, but smaller and shorter rise was noted. Owing to lack of detail regarding the "unit" adopted it is impossible to compare directly our figures with those of Saidl. It may be added that a response similar to those of Table II has been obtained by us with urine from normal pregnant women.

SUMMARY.

1. The operation of bilateral oophorectomy in pregnancy does not bring about any change in the anterior hypophyseal hormone content of the urine.

2. The oestrone content of the urine is definitely decreased.

It is a pleasure to express our gratitude to Mr Aleck Bourne for his courtesy in drawing our attention to this case and permitting us to publish our results, and to Mr E. R. E. Spence for his careful work in the animal experiments.

REFERENCES.

Amati (1928). *Zentr. Gyn.* **52**, 2639.
Saidl (1932). *Časopis Lékařu Českych*, **71**, 18.
Szarka (1930). *Zentr. Gyn.* **54**, 2211.
Waldstein (1929). *Zentr. Gyn.* **53**, 1305.

XXXV. STUDIES IN THE METABOLISM OF THE STRICT ANAEROBES (GENUS *CLOSTRIDIUM*).

II. THE REDUCTION OF PROLINE BY *CL. SPOROGENES*.

By LEONARD HUBERT STICKLAND.[1]

From the Biochemical Laboratory, Cambridge.

(*Received December 19th, 1934.*)

The first paper of this series [Stickland, 1934] showed that washed suspensions of *Cl. sporogenes* are able to oxidise certain amino-acids (*e.g.*, *d*-alanine, *d*-valine, *l*-leucine, *l*-histidine) and reduce certain others (glycine, *l*-proline, *l*-hydroxyproline) and are able also to catalyse direct oxidation-reduction reactions between pairs of these amino-acids. The study of the chemistry of these reactions obviously involves the use of two substrates simultaneously, so that there is difficulty in deciding from which of these substrates the products have arisen. If, however, the products of reaction of any one amino-acid can be determined, the rest of the information required will follow from the analysis of the products of reaction of the various pairs. The oxidation of alanine by cresyl blue, described in the previous paper, would serve as a starting point, but it is impossible by this method to oxidise sufficient alanine to permit of the identification and estimation of the products. Eventually for various reasons the reduction of *l*-proline was chosen as a starting point, and the present paper deals with this subject.

EXPERIMENTAL.

Preparation of bacterial suspensions.

Suspensions of *Cl. sporogenes* were prepared as before [Stickland, 1934] by growing the bacteria for 40 hours anaerobically on tryptic digest of caseinogen, centrifuging and washing twice with Ringer's solution.

Preliminary experiments.

It was first shown that *l*-proline is not deaminated during reduction. A series of Thunberg tubes (usually five tubes) was prepared containing 0·5 ml. *M*/10 *d*-alanine, 0·5 ml. *M*/5 *l*-proline, 0·5 ml. *M*/2 phosphate buffer p_H 7·5 and 1·0 ml. suspension of *Cl. sporogenes*, and another series of controls with neither amino-acid. These were all evacuated thoroughly and incubated at 40°. After various intervals of time pairs of tubes, one from each series, were taken and the ammonia was estimated in 2 ml. of their contents. It was found that ammonia production ceased when an amount equivalent to the alanine present had been liberated. (See Fig. 1 and Table I.)

In the previous paper it was shown that alanine is deaminated during oxidation, so these results show that proline is not deaminated during reduction.

The reduction of proline must therefore take place by a simple opening of the ring, giving an amino-*n*-valeric acid. The amino-group might equally well occupy the α- or δ-position, according to the point at which the ring was opened (see

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[1] Beit Memorial Fellow.

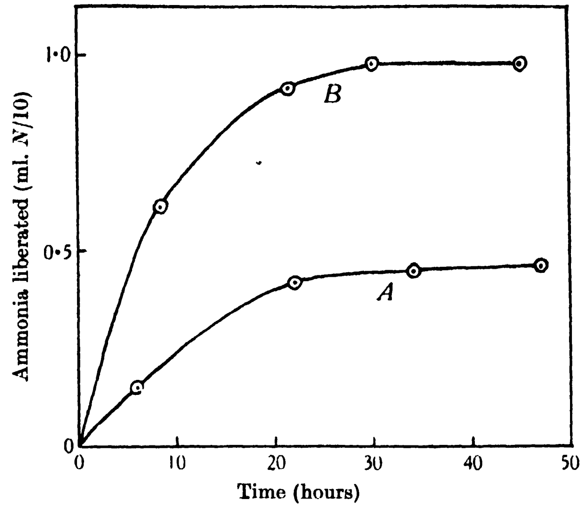
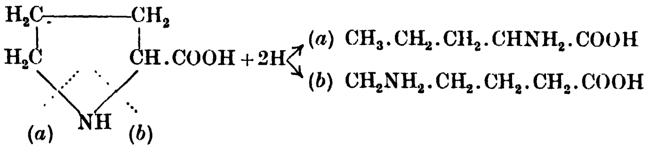


Fig. 1. The course of liberation of ammonia from
A. 0·5 ml. *M*/10 *d*-alanine + 0·5 ml. *M*/5 *l*-proline.
B. 0·5 ml. *M*/5 *d*-alanine + 0·5 ml. *M*/2·5 *l*-proline.

Table I. *Ammonia production from 1 mol. alanine + 2 mols. proline.*

Alanine	Proline	Ammonia found (ml. *M*/10) (corrected for blank)	Ammonia as % of theoretical from alanine
0·5 ml. *M*/10	0·5 ml. *M*/5	0·46	92
,,	,,	0·45	90
0·5 ml. *M*/5	0·5 ml. *M*/2·5	0·98	98
,,	,,	0·99	99

equations). Results reported by Neuberg [1911] and Ackermann [1911] on the action of mixed putrefactive bacteria on proline to give δ-amino-*n*-valeric acid make it likely that in the present case also the ring is opened to give the δ-amino compound.



$$\text{CH.COOH} + 2\text{H} \to \begin{cases} (a)\ CH_3.CH_2.CH_2.CHNH_2.COOH \\ (b)\ CH_2NH_2.CH_2.CH_2.CH_2.COOH \end{cases}$$

*Synthesis of δ-amino-*n*-valeric acid.*

Piperidine was benzoylated by the method of Marvel and Lazier [1929]. Oxidation of benzoylpiperidine to benzoyl-δ-amino-*n*-valeric acid and hydrolysis of this to δ-amino-*n*-valeric acid hydrochloride were carried out by the method of Schotten [1884]. The nitrogen content of the product dried *in vacuo* was 9·20 % ($C_5H_{12}O_2NCl$ requires N 9·14 %).

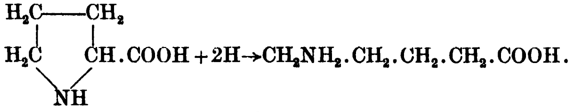
Preparation of α-naphthyl isocyanate *derivative of δ-amino-*n*-valeric acid.* The reaction with α-naphthyl *iso*cyanate was carried out exactly as described by Neuberg and Rosenberg [1905]. The product decomposed at 195–196° and melted at 231° [*cf.* Keil, 1932]. (Found: N 9·9 %; $C_{16}H_{18}O_3N_2$ requires N 9·8 %.)

*Isolation of the α-naphthyl isocyanate derivative of δ-amino-*n*-valeric acid from the products of the reaction between alanine and proline.*

A large (75 ml.) Thunberg tube was prepared containing 8 ml. *M*/5 *d*-alanine, 8 ml. *M*/2·5 *l*-proline (368 mg.), 8 ml. *M*/2 phosphate buffer p_H 7·5 and 16 ml. of a suspension of *Cl. sporogenes*. To enable the course of the reaction to be followed, two sets of small Thunberg tubes were set up containing (*a*) 0·5 ml. *M*/5 alanine, 0·5 ml. *M*/2·5 proline, 0·5 ml. buffer and 1·0 ml. suspension of bacteria, and (*b*) 1·0 ml. water, 0·5 ml. buffer and 1·0 ml. suspension. All the tubes were evacuated and incubated at 40°. Pairs of the small tubes (one of (*a*) and one of (*b*)) were taken from the incubator from time to time, and the ammonia was estimated in them in the usual way. It was found that in 22½ hours 99 % of the nitrogen of the alanine had been liberated as ammonia.

The contents of the large tube, after the greater part of the bacteria had been removed by centrifuging, were strongly acidified with hydrochloric acid and evaporated to dryness *in vacuo*. From the dry residue the hydrochloride of δ-amino-*n*-valeric acid was extracted with hot 97 % alcohol (once with 30 ml. and twice with 10 ml.), and the combined filtered extracts were evaporated to dryness *in vacuo*, taken up in 15 ml. of water and filtered. The solution was neutralised with *N* NaOH, made alkaline by the addition of a further 5 ml. of NaOH, and freed from a trace of ammonia by a current of air. The solution was now treated with 0·5 g. of α-naphthyl *iso*cyanate and shaken for one hour. The precipitate of di-α-naphthylurea was filtered off and the filtrate acidified with hydrochloric acid and kept at 2° for several hours. The precipitated derivative was now filtered off and washed, redissolved in hot dilute ammonia and reprecipitated with dilute acetic acid. After two such reprecipitations the product was dried and weighed. At this stage the yield was 0·52 g., which is equivalent to 58 % of the proline originally taken.

After three recrystallisations from dilute alcohol, the substance alone and in admixture with synthetic α-naphthylcarbamido-*n*-valeric acid decomposed at 190–191° and melted at 231°. (Found: N 9·7 %; $C_{16}H_{18}O_3N_2$ requires N 9·8 %.) The decomposition temperature of this product is rather low, but the melting-point, mixed melting-point with the synthetic substance and nitrogen content afford satisfactory proof that *Cl. sporogenes* reduces *l*-proline to δ-amino-*n*-valeric acid according to the equation



SUMMARY.

Washed suspensions of *Cl. sporogenes* reduce *l*-proline, at the expense of the oxidation of alanine, to δ-amino-*n*-valeric acid.

REFERENCES.

Ackermann (1911). *Z. Biol.* **57**, 104.
Keil (1932). *Z. physiol. Chem.* **207**, 248.
Marvel and Lazier (1929). *Organic Syntheses*, **9**, 16.
Neuberg (1911). *Biochem. Z.* **37**, 490.
—— and Rosenberg (1905). *Biochem. Z.* **5**, 456.
Schotten (1884). *Ber. deutsch. chem. Ges.* **17**, 2544.
Stickland (1934). *Biochem. J.* **28**, 1746.

XXXVI. DILATOMETRIC DETERMINATION OF THE RELATIVE DIGESTIBILITY OF PROTEINS.

By HEMMIGE BILIGIRIRANGACHAR SREERANGACHAR
AND MOTNAHALLI SREENIVASAYA.

From the Department of Biochemistry, Indian Institute of Science, Bangalore.

(*Received November 7th, 1934.*)

Digestibility of proteins is usually determined by feeding tests employing either the method of Mitchell [1924] or the alternative method of Osborne *et al.* [1919]. Experiments with animals are time consuming and cumbersome as compared with the *in vitro* studies carried out by Waterman and his colleagues [Waterman and Johns, 1921; Waterman and Jones, 1921; Jones and Waterman, 1923]. According to them the relative digestibilities of the proteins of a food material can be directly and satisfactorily determined by enzymic digestions *in vitro.*

In vitro studies offer a convenient method of investigating the digestion of proteins and starches as influenced by cooking, addition of salts and other activating or inhibiting agencies. The increased digestibility of phaseolin on cooking [Waterman and Jones, 1921], the relative indigestibility of arachin [Jones and Waterman, 1922], the inhibiting effect of gossypol on the digestibility of cotton seed proteins [Jones and Waterman, 1923], the influence of fats on protein digestion in general [Maughan, 1926] have all been studied by *in vitro* methods. These methods are particularly suitable for investigating the influence of active principles like glucosides and alkaloids on the digestion of proteins.

All *in vitro* experiments involve the enzymic digestion of the material under certain standardised conditions of reaction and temperature, so chosen that they approximate as closely as possible to the condition obtaining in the animal. The course of digestion is followed mostly by chemical methods involving a determination of either the amino-nitrogen released by van Slyke's method or the total nitrogen in the filtrate after removing the undigested protein by precipitants like trichloroacetic and phosphotungstic acids. In a few investigations the rate at which a particular amino-acid is liberated during digestion has been adopted as a measure of digestibility. Jones and Gersdorff [1933] have thus followed the peptic and acid digestions of caseinogen by estimation of the liberated cystine.

The relative digestibility of proteins can also be studied by any of the physical methods whose experimental values are proportional to the extent of digestion. Thus, the dilatometric estimation offers a convenient method of studying the relative digestibility of proteins, since it has been shown that the release of amino-nitrogen is proportional to the volume change accompanying the enzymic digestion [Sreenivasaya *et al.*, 1934]. The quantity of material required for an investigation of this type is comparatively small, the results are quickly and easily reproducible and the digestibility of a number of proteins with reference to that of a standard protein like caseinogen can be simultaneously determined.

EXPERIMENTAL.

The present communication relates to a study of the digestibility of three proteins, gelatin (Gold-label), and the globulins of *Phaseolus mungo* and *Dolichos lablab*, as compared with the digestibility of "Hammersten's casein." Since the principal object of the investigation was to demonstrate the adaptability of the dilatometric method for *in vitro* digestion studies, all the digestions were carried out with Pfanstiehl's trypsin at 30° and at p_H 7·7 employing Sørensen's phosphate buffer.

The globulins were extracted from the respective pulses with 5 % sodium chloride solution, the clear extract was dialysed against distilled water in cellophane bags, and the precipitated globulins were recovered by centrifuging. The wet precipitate was dissolved directly in the phosphate buffer and after a preliminary determination of the protein content (N × 6·25) it was adjusted to yield a 1 % protein solution. 50 ml. of this solution and 10 ml. of a 1 % trypsin solution were employed for the dilatometric determination. The reaction was conducted simultaneously in another flask and the digestion followed independently by a determination of the amino-nitrogen by van Slyke's method at definite intervals. The experimental procedure employed in this study is the differential method fully described in one of our earlier communications (1932).

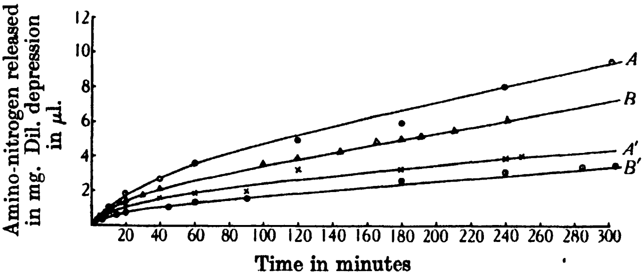


Fig. 1. *A*, *Phaseolus mungo* protein amino-nitrogen; *B*, *Phaseolus mungo* protein dil. depression; *A'*, *Dolichos lablab* protein amino-nitrogen; *B'*, *Dolichos lablab* protein dil. depression.

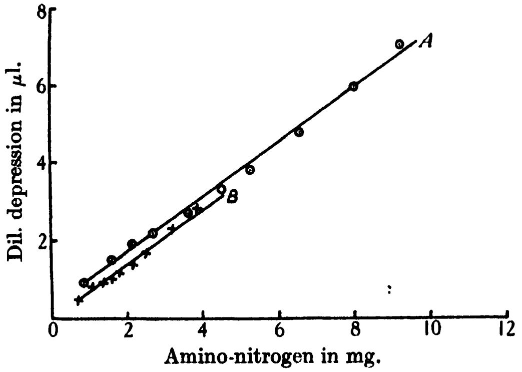


Fig. 2. *A*, *Phaseolus mungo* protein; *B*, *Dolichos lablab* protein.

The course of digestions of the globulins from *Phaseolus mungo* and *Dolichos lablab* has been followed both by the dilatometric and van Slyke's methods and the results are graphically represented in Fig. 1. Fig. 2 gives the correlation between the dilatometric depression and amino-nitrogen indicating thereby that the dilatometric depressions are proportional to the release of amino-nitrogen during the digestion. That a similar proportionality also exists in the case of caseinogen and gelatin digestions has been established in one of our earlier communications.

DISCUSSION.

If X is the amount of amino-nitrogen that is released during the digestion of a protein (expressed as A g. of total nitrogen) under certain standardised experimental conditions, then the digestibility K of the protein is given by $K = X \times 100$. The digestibilities of caseinogen, gelatin and the *Phaseolus mungo* and *Dolichos lablab* globulins for different intervals of time, are given in Table I.

Table I.

Time in hours ...	1	2	3	4	5
Caseinogen	11·1	14·8	16·9	18·0	18·6
Gelatin	6·7	9·1	10·4	10·9	11·3
P. mungo	5·7	8·3	10·3	12·6	14·5
D. lablab	2·9	4·0	5·1	6·1	6·7

The digestibility appears to increase with time (Table I) reaching a fairly steady value in about 4–5 hours. For comparative studies of digestibility a 4–5 hour period can therefore be fixed as a standard.

Since the dilatometric depressions V have been shown to be proportional to the amino-nitrogen release X during the digestion, the ratio between the digestibility of a protein and that of the caseinogen standard will be equal to the ratio of either the amounts of amino-nitrogen released or the depressions that accompany the digestion. The relative digestibility is therefore given by either

$$K_1 = \frac{\text{NH}_2\text{ (protein) } X_1}{\text{NH}_2\text{ (caseinogen) } X_2} \times 100$$

or

$$K_2 = \frac{\text{Dil. depression } V_1 \text{ (protein)}}{\text{Dil. depression } V_2 \text{ (caseinogen)}} \times 100.$$

The values of K_1 and K_2 for two periods of digestion are given in Table II and there is a fair agreement between the two values indicating the usefulness and reliability of the dilatometric method in assaying the relative digestibility of proteins.

Table II.

	Gelatin		*P. mungo*		*D. lablab*	
	K_1	K_2	K_1	K_2	K_1	K_2
4 hours	60	58	70	65	34	32
5 hours	60	59	78	74	36	35

The results indicate that *Phaseolus* protein is digested much more easily (about twice) than that of *Dolichos*, and are in agreement with the observations of Desai *et al.* [1931–32] made by feeding tests of these proteins.

SUMMARY.

1. The globulins from *Phaseolus mungo* and *Dolichos lablab* have been subjected to tryptic digestion and the course of reaction followed both by the dilatometric and van Slyke's methods; it is found that the dilatometric depression obtained in each case is proportional to the release of amino-nitrogen.

2. It is shown that dilatometric depressions can be directly taken as a measure of digestibility and that the ratio of the depressions observed with the experimental protein and with the standard caseinogen represents the relative digestibility.

Our best thanks are due to Mr B. N. Sastri for his kind help in the preparation of the globulins and for his many helpful suggestions.

REFERENCES.

Desai, Narayana and Neogi (1931–32). *Indian J. Med. Res.* **19**, 1041.
Jones and Gersdorff (1933). *J. Biol. Chem.* **101**, 657.
Jones and Waterman (1922). *J. Biol. Chem.* **52**, 357.
—— —— (1923). *J. Biol. Chem.* **56**, 501.
Maughan (1926). *Biochem. J.* **20**, 1046.
Mitchell (1924). *J. Biol. Chem.* **58**, 873.
Osborne, Mendel and Ferry (1919). *J. Biol. Chem.* **37**, 223.
Sreenivasaya, Sastri and Sreerangachar (1934). *Biochem. J.* **28**, 351.
—— and Sreerangachar (1932). *J. Indian Inst. Sci.* **15** A, 17.
Waterman and Johns (1921). *J. Biol. Chem.* **46**, 9.
—— and Jones (1921). *J. Biol. Chem.* **47**, 285.

XXXVII. AN ISOTHERMAL CALORIMETER[1].

BY HEMMIGE BILIGIRIRANGACHAR SREERANGACHAR
AND MOTNAHALLI SREENIVASAYA.

From the Department of Biochemistry, Indian Institute of Science, Bangalore.

(*Received November 26th, 1934.*)

AMONG the existing isothermal calorimeters for the measurement of the heat liberated during reaction, dilution or solution are the compensation device of Wasserman [1930], where the heat liberated is balanced by the cooling caused by diluting a concentrated solution of ammonium nitrate, and the isothermal calorimeter of Hirobe [1926] in which the temperature change is prevented by adding simultaneously with the progress of the reaction, the necessary quantity of an indifferent substance at a different temperature. Coon and Daniels [1933] recommend a calorimeter particularly suitable for measuring the heat changes accompanying prolonged reactions like the hydrolysis of ethyl acetate and the fermentation of yeast.

While the above instruments are cumbersome and require skilled and elaborate manipulation, the original ice calorimeter of Bunsen [1870] remains one of the most simple and successful devices in the field. For biochemical reactions Bunsen's calorimeter cannot be employed since the reactions proceed extremely slowly at 0°, but if ice could be replaced by a substance melting at 25–30° the instrument might find extended application in biochemistry. The substance chosen should satisfy the following requirements: (1) a reproducible and sharp melting-point, (2) stability at the temperature of melting without any decomposition on repeated meltings and coolings, (3) indifference towards the mercury used in the capillary, (4) appreciable volume change during the melting of the solid and (5) low latent heat of fusion contributing towards a greater sensitivity of the instrument.

Diphenylmethane, which has been tried in our present study, meets most of these requirements, in that it is a stable hydrocarbon melting at 24·5° and has a latent heat of fusion of 26 cal./g. *p*-Bromotoluene (M.P. 28·5°), *tert*butyl alcohol (M.P. 25°), *iso*quinoline (M.P. 24·5°) and diphenylether (M.P. 28°) are suggested as possible alternatives.

The instrument and its manipulation.

The instrument (Fig. 1*a*) (kindly made to our design by Messrs Greiner and Freidrichs) consists of a reaction tube *A* surrounded by a bulb *B* which, in turn, is thermally insulated by a vacuum jacket *C*. The tube *D* leading to the bottom of the bulb *B*, communicates with the capillary *L* through the standard ground glass joint *J*. The capillary tube leading out of the apparatus is bent at right angles towards its extreme end and can be made whenever necessary to dip into mercury contained in a crucible.

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[1] A preliminary report of this paper was presented before the Joint Session of the Society of Biological Chemists, India, and the Association of Economic Biologists held at Coimbatore in October 1932 (*Proc. Soc. Biol. Chemists, India*, 1932, **18**). It formed part of a thesis submitted by one of the authors (H. B. S.) for the Associateship of the Indian Institute of Science, Bangalore. While this paper was being prepared for publication, there appeared a note on this subject by Schukarew *et al.* [1934].

The bulb *B* is evacuated and filled with diphenylmethane. The tube *D* is then filled with mercury and the capillary inserted at the ground joint when the column of mercury fills the capillary. When the instrument is not in use the capillary end is kept dipping into mercury so that changes in surrounding temperature will not effect a break in the mercury column and necessitate refilling of the apparatus.

The instrument after filling is placed in a Dewar flask containing melting diphenylmethane. Initially part of the liquid inside the calorimeter is frozen by evaporating dry ether in the reaction tube *A*, by means of a current of dry air. The calorimeter is then allowed to remain in the Dewar flask containing melting diphenylmethane for 48–72 hours so as to attain thermal equilibrium with the surroundings.

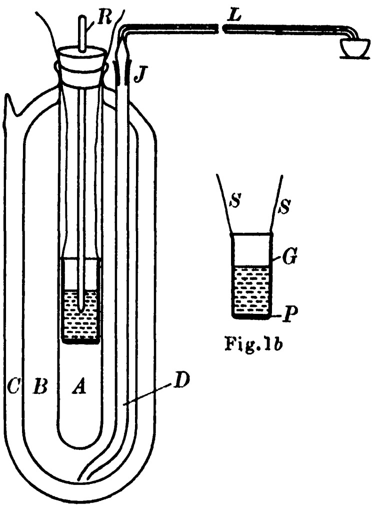

Fig. 1*b*

Fig. 1*a*

Experimental procedure.

Before the commencement of an experiment, 1 ml. of pure dry ether is introduced into the reaction tube and evaporated by a current of dry filtered air. By this means, a uniform incrustation of solid diphenylmethane is formed on the outer walls of the reaction tube. The mercury meniscus in the capillary can be adjusted to any desired position by withdrawing the mercury cup at the end of the capillary and continuing the operation of cooling. The interior of the reaction tube is then dried by means of a swab of clean cotton wool.

The "housing" of the reagents. The "housing" of the two reacting liquids separately in the calorimeter offered considerable difficulty. Before mixing they should be kept separate inside the calorimeter and allowed to attain the temperature of the melting diphenylmethane. The mixing operation itself should not involve any disturbance in the thermal equilibrium of the whole system. After several trials, the arrangement illustrated in Fig. 1*b* was found to be satisfactory. One of the solutions is introduced directly into the reaction tube while the other is contained in a separate small glass tube *G* closed at one end with a thin layer of paraffin wax *P* and suspended in the reaction tube by means of a non-conducting string *S* of cotton. A glass rod *R* which passes through the cork closing the reaction tube dips into the liquid contained in *G*. The projection of the glass rod over the cork is covered with felt to minimise transference of heat from outside to inside. After the calorimeter has attained thermal equilibrium as shown by the steadiness of the creep of the mercury column, the glass rod is gently pushed in, breaking the paraffin bottom of the glass tube *G* and effecting a mixture of the two liquids. That the operation of mixing itself does not lead to any detectable disturbance in the thermal equilibrium of the calorimeter can be shown by mixing distilled water in the reaction tube with water in the suspended glass tube; the position of the mercury column remains undisturbed.

The heat of reaction produced is measured by the shift in the mercury meniscus along the capillary. Before an experiment is begun, the rate of flow of mercury is determined. When this is minimum and fairly steady, the liquids are mixed and measurements continued. The shift of mercury column due to the heat of reaction is obtained by allowing for the already determined creep.

Fig. 2 gives a graphical representation of the course of heat evolution during a few typical experiments. Curve *A* is characteristic of all reactions like the neutralisation of HCl with NaOH wherein the heat liberation is instantaneous.

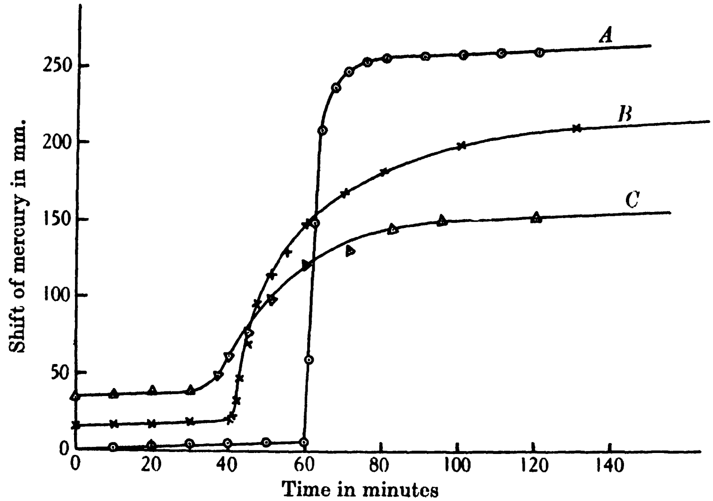

Fig. 2. *A*, neutralisation of HCl with NaOH. *B*, *C*, quinol-peroxidase system (two concentrations of substrate.)

B and *C* represent the curves characteristic of reactions, particularly enzymic reactions, where heat generation is gradual. In all three cases there is first a flat portion of the curve corresponding to a uniform change in the initial conditions of the calorimeter, then either a steep or a gradual rise corresponding to either a rapid or slow liberation of heat due to reaction and finally again, a flat portion showing the completion of the reaction and the attainment of a state of thermal equilibrium. By the use of a thermostat maintained at the melting temperature of diphenylmethane, more steady conditions could be obtained in these various stages.

Calibration of the instrument.

The calorimeter is calibrated by conducting standard reactions which evolve known amounts of heat. Dilution of sulphuric acid, neutralisation of hydrochloric acid with sodium hydroxide and double decomposition of barium chloride and sodium sulphate were all carried out in the calorimeter and the results obtained are given in Table I. The heats liberated in each case have been calculated from the values given in Standard Physical Tables.

It will be observed from the results in Table I that the calorimeter gives reliable and concordant results as tested by three independent reactions. The average quantity of heat corresponding to 1 mm. shift of mercury column is 0·063 cals.

Table I.

Reaction	Mercury shift mm.	Heat liberated cals.	Heat liberated per mm. shift of mercury cals.
1. Dilution of 49 % sulphuric acid	153		0·063
2 ml. of acid + 1 ml. water	155	9·58	0·062
	154		0·062
Mean	154		0·062
2. Neutralisation of HCl with NaOH	254		0·066
2 ml. 0·62N acid +	252	16·76	0·067
2 ml. 0·65N alkali	253		0·066
Mean	253		0·066
3. Double decomposition of $BaCl_2$ and Na_2SO_4	25		0·059
2 ml. 2 % Na_2SO_4 +	25	1·48	0·060
2 ml. 3 % $BaCl_2$	24		0·061
Mean	25		0·060

Application to biochemical reactions.

The heat changes accompanying the enzymic hydrolysis of a few substrates, urea, sucrose, amygdalin and quinol, have been investigated in the calorimeter. While the amounts of heat in the cases of urease-urea, invertase-sucrose and emulsin-amygdalin systems, as determined by our present calorimeter are too small to be accurately estimated, the instrument has shown itself suitable for a study of the peroxidase-quinol system (Table II).

Table II. *Peroxidase-quinol.*

Quinol mg.	Quantity of enzyme	Shift of mercury column mm.	Heat liberated per g. of substrate cals.
91·0	2 ml. potato extract + 1 ml. 1 % H_2O_2	93·0	64·4
81·6	,,	90·5	70·4
80·2	,,	89·0	69·8

The mean value obtained for the liberation of heat by the peroxidase-quinol system is 68·2 cals. per g. of quinol. In the case of the other three systems, urease-urea, emulsin-amygdalin and invertase-sucrose, the heat changes involved have been found to be too small to be measured with adequate accuracy. If, for example, the value for sucrose hydrolysis be accepted as 11·21 cals. per g. of sucrose, as determined by Brown and Pickering [1897] and Dixon and Ball [1920], then the expected value under the conditions of our experiment should correspond to a shift of 9 mm. in the mercury column which can be detected by the present calorimeter but not measured with sufficient accuracy. Obviously, those reactions which are accompanied by heat changes corresponding to shifts of less than 15–20 mm. of mercury cannot be investigated by the instrument in its present form. By employing a capillary with a smaller bore, the sensitivity of the calorimeter can be increased and reactions involving smaller heat changes studied. An attempt in this direction is being made. The calorimeter is now being employed for measuring the heat liberated during the respiration of small insects, germination of seeds and a few typical fermentations.

SUMMARY.

1. An isothermal calorimeter using diphenylmethane, which is very simple in operation, is described.

2. The calorimeter has been calibrated with the help of the following standard reactions: (*a*) dilution of sulphuric acid, (*b*) neutralisation of HCl with NaOH, and (*c*) double decomposition of Na_2SO_4 and $BaCl_2$.

3. The heat changes accompanying a few enzymic reactions have been investigated; the peroxidase-quinol system yields an average value of 68·2 cals. per g. of substrate.

4. The possibility of employing the calorimeter for the investigation of other biological reactions, *e.g.* respiration, germination and fermentation, is indicated.

In conclusion, we wish to express our grateful thanks to Mr G. Gundu Rao, Chemist, Mysore Sugar Factory, for many helpful suggestions in the course of the work and to Dr M. A. Govinda Rau, Department of Physics, Indian Institute of Science, for his constructive criticisms in the preparation of the paper.

REFERENCES.

Brown and Pickering (1897). *J. Chem. Soc.* **71**, 783.
Bunsen (1870). *Poggendorf's Ann.* **141**, 1.
Coon and Daniels (1933). *J. Physical Chem.* **37**, 1.
Dixon and Ball (1920). *Sci. Proc. Roy. Dub. Soc.* **16**, 153.
Hirobe (1926). *J. Faculty Sci. Imp. Univ. Tokyo*, **1**, 155.
Schukarew, Krivobabko and Schukarewa (1934). *Physik. Z. Sowjetunion*, **5**, 722.
Wasserman (1930). *Z. physikal. Chem.* **146**, 409.

XXXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLI. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH. II. THE MOLECULAR CONSTITUTION OF CAROLIC AND CAROLINIC ACIDS.

BY PERCIVAL WALTER CLUTTERBUCK, HAROLD RAISTRICK AND FRITZ REUTER.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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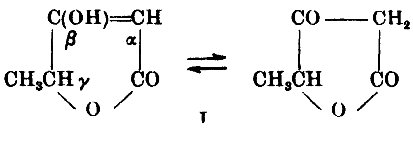
IN a recent communication [Clutterbuck *et al.*, 1934] it was shown that *Penicillium Charlesii* G. Smith, a new species of *Penicillium* isolated by our late colleague Mr J. H. V. Charles from mouldy Italian maize, produced from glucose a polygalactose and a polymannose together with a number of hitherto undescribed organic acids. Of these acids carolic and carolinic acids were obtained in the largest yields. It is the purpose of this paper to describe the investigation of the molecular constitutions of these two acids.

Carolic acid gives an orange $FeCl_3$ colour but does not give an immediate violet colour with $NaNO_2$. In aqueous solution it is acid to Congo red and titrates in the cold as a monobasic acid, the titration not being materially altered by gently warming with excess $N/10$ NaOH. The acid does not however contain any active hydrogen as determined in dry anisole by the Zerewitinoff method, whilst it contains one active hydrogen when determined in pyridine (Roth). It appears therefore that carolic acid contains a non-lactonic ring which opens up almost completely during titration in the cold in aqueous solution, whilst in pyridine one ketonic group becomes enolised.

Carolinic acid gives with $FeCl_3$ an immediate precipitate, soluble in excess to a bright orange coloured solution, but does not give an immediate $NaNO_2$ colour. The acid turns Congo red blue, titrates sharply in the cold as a dibasic acid and contains 2 atoms of active hydrogen as determined in dry anisole or pyridine by the Zerewitinoff method (Roth).

Both carolic and carolinic acids on boiling with $2N$ H_2SO_4 undergo immediate hydrolysis, carolic acid giving quantitatively 1 molecule each of acetoin, CO_2 and butyrolactone, whilst carolinic acid gives 1 molecule each of acetoin, CO_2 and succinic acid.

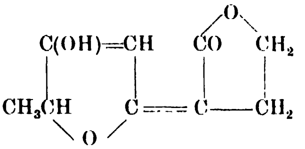
Both carolic and carolinic acids on brominating in dilute acetic acid solution give *d*-α-bromo-γ-methyltetronic acid ($C_5H_5O_3Br$) which on catalytic reduction with palladium and hydrogen very readily loses its bromine to give *l*-γ-methyltetronic acid (I).

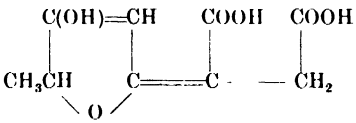


I

On hydrolysis with $2N$ H_2SO_4 (I) quantitatively gives 1 molecule each of acetoin and CO_2 and it is therefore reasonable to suppose that the acetoin and CO_2 obtained on hydrolysis of carolic and carolinic acids arise from the γ-methyltetronic acid nucleus present in these acids and separated from them on bromination. Carolic and carolinic acids may therefore be represented as arising from the condensation of 1 molecule of *l*-γ-methyltetronic acid with 1 molecule butyrolactone and 1 molecule succinic acid respectively, in each case with the elimination of 1 molecule of water.

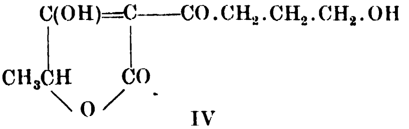
Various methods of postulating these condensations might be suggested. It is improbable that an esterification occurs at the enolised β-carbonyl group of the γ-methyltetronic acid ring since both carolic and carolinic acids on reduction with Pd and hydrogen give products which on hydrolysis still decarboxylate (though much more slowly than the parent acids) but no longer break down into two C_4 compounds. Since both the acids themselves and the reduced acids give hydroxyketones on hydrolysis it seems unlikely that the β- and γ-carbons of the γ-methyltetronic acid ring are involved in the condensation. On the other hand condensation might take place at the lactonic carbonyl group of the γ-methyltetronic acid ring, when carolic and carolinic acids would have structures II and III respectively.

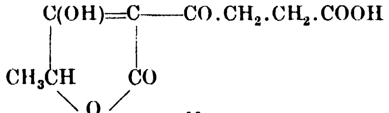

II


III

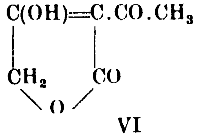
An example of this type of structure is given by Fittig and Ström's [1892] dibutyrolactone. It will be shown however that formulae of this type cannot explain a number of properties of these acids.

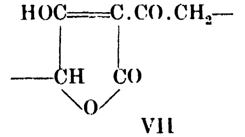
Alternatively linkage may occur at the α-CH_2 group, when carolic acid ($+H_2O$) and carolinic acid would have the structures IV and V respectively.


IV


V

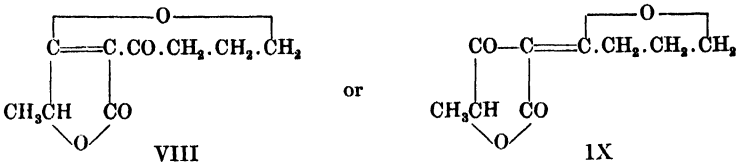
An example of this type of structure is given in Benary's [1910] α-acetyltetronic acid (VI) which has the grouping VII, in common with carolic and carolinic acids.


VI


VII

As a result of the absolute analogy in properties and absorption spectra between α-acetyltetronic and carolic and carolinic acids and their derivatives, the ketonic formulae IV and V have now been adopted for the hydrated form of carolic acid ($C_9H_{10}O_4+H_2O$) and for carolinic acid respectively.

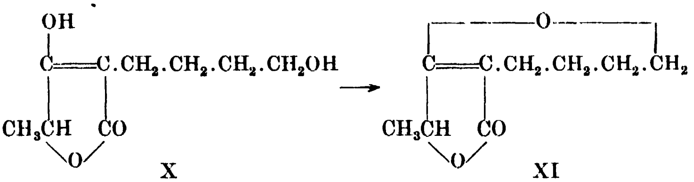
The structural formula of carolic acid ($C_9H_{10}O_4$) then requires the removal of the elements of water from IV. This may be done in two ways giving formulae VIII and IX.



VIII IX

No decisive chemical evidence can be advanced for these formulae, but VIII is to be preferred. Thus it seems probable that the 7-membered ring of VIII would be more labile than the 5-membered ring of IX and would therefore more readily explain the addition of H_2O by mere solution in water. Moreover some heterocyclic structures containing more than six members are known, and on solution in water do form equilibrated systems [*cf. e.g.*, Helferich and Schäfer, 1924].

Further, it should be noted that reduced carolic acid ($C_9H_{14}O_4$), which is a liquid titrating as a monobasic acid, and which is shown later to have the structure X, readily loses one molecule of H_2O on distillation *in vacuo* to give a crystalline neutral compound $C_9H_{12}O_3$ (XI).



X XI

This reaction cannot lead to the formation of a 5-membered ring but must give the same 7-membered ring as is postulated above in the formula VIII for carolic acid with the exception that the CO group immediately outside the tetronic acid ring in carolic acid is reduced to a CH_2 group. In order to account for the single active hydrogen in carolic acid when dissolved in pyridine it is postulated that the $COCH_2$ group of the 7-membered ring undergoes enolisation to $C(OH)=CH$. A number of similar enolisations in pyridine have recently been reported [Ishikawa and Kojima, 1934].

It remains now to be shown that these constitutional formulae are in full agreement with all the known properties of carolic, carolinic and α-acetyltetronic acids.

(*a*) *General properties of carolic, carolinic and α-acetyltetronic acids.*

The above formulae for carolic and carolinic acids would obviously permit optical activity. Carolic and carolinic acids give with excess of $FeCl_3$ characteristic orange colours indistinguishable from that obtained with α-acetyltetronic acid. In view of the substitution of the α-carbon of the tetronic acid ring we should not expect any of them to give an immediate $NaNO_2$ colour, as is indeed the case. The fact that all three acids can be recovered unchanged after boiling with acetyl chloride is explained by the fact that the only hydroxyl group is of the keto-enol type and is reconverted into the ketonic form in presence of this reagent. The acids also do not give benzoyl, *p*-nitro- or *p*-bromo-benzoyl derivatives, the material being recovered unchanged. Assuming the above configuration, carolic acid would not contain any active hydrogen in dry anisole whereas carolinic acid would contain two active hydrogens. Assuming also that carolic acid adds 1 molecule of water in aqueous solution, it would then become a monobasic acid, the three carbonyl groups surrounding the hydrogen atom of the α-carbon of the tetronic acid ring imparting to it strongly acidic properties.

(*b*) *Bromination of carolic, carolinic and α-acetyltetronic acids.*

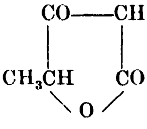
Considerable advance in our knowledge of the structure of these acids has been made by a detailed study of the products of bromination under varying conditions. Thus carolic acid in glacial acetic acid solution absorbs 1 molecule of bromine and gives 1 molecule of HBr and 1 molecule of monobromocarolic acid, $C_9H_9O_4Br$, containing its bromine atom in the butyrolactone half of the molecule since on hydrolysis it gives acetoin, CO_2 and bromobutyrolactone. In dilute acetic acid solution, on the other hand, carolic acid reacts in two ways:

$$\text{I.}\quad C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_4O_3Br_2 + C_4H_6O_2 + 2HBr$$

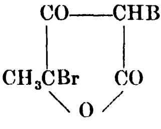
$$\text{II.}\quad C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_5O_3Br + C_4H_5O_2Br + 2HBr$$

In reaction I the products are a C_5 dibromide and butyrolactone, and in reaction II a C_5 monobromide and a bromobutyrolactone. In aqueous solution reaction I occurs, and in 50 % acetic acid solution both reactions occur but reaction II predominates. If carolic acid is, on the other hand, allowed first to react with 1 molecule of bromine in glacial acetic acid solution, then diluted to about 50 % acetic acid and a further molecule of bromine added, the products are exclusively the C_5 monobromide, bromobutyrolactone and HBr.

The C_5 monobromide has been shown to be *d*-α-bromo-γ-methyltetronic acid (XII) whilst the dibromide is very probably *d*-αγ-dibromo-γ-methyltetronic acid (XIII). Both of these bromides on catalytic reduction readily give the same *l*-γ-methyltetronic acid (I).



XII



XIII

With bromine in aqueous or 50 % acetic acid solution, carolinic acid reacts, like carolic acid, in two ways, the same *d*-α-bromo-γ-methyltetronic acid being obtained, whilst succinic and probably bromosuccinic acid replace butyrolactone and bromobutyrolactone. The identity of the two samples of *d*-α-bromo-γ-methyltetronic acid from carolic and carolinic acids respectively was proved by M.P., mixed M.P., titration and conversion into *l*-γ-methyltetronic acid and its dinitrophenylhydrazone.

It appears therefore that during bromination in dilute acetic acid solution the α-side chains are replaced by bromine. An exactly analogous behaviour was observed with synthetic α-acetyltetronic acid, which on bromination in dilute acetic acid gave α-bromotetronic acid.

(*c*) *Hydrolysis of carolic, carolinic and α-acetyltetronic acids.*

All three acids undergo hydrolysis with $2N$ H_2SO_4 at comparable rates, each giving 1 molecule of CO_2, carolic acid giving in addition 1 molecule acetoin and 1 molecule γ-hydroxybutyric acid (butyrolactone), carolinic acid giving 1 molecule acetoin and 1 molecule succinic acid, and α-acetyltetronic acid giving 1 molecule acetol and 1 molecule acetic acid. This rather striking property is undoubtedly due to the structure VII which they all have in common and which on opening the tetronic acid ring represents them as derivatives of diacetylacetic acid.

It will be noted, on the other hand, that formula II for carolic acid, obtained by analogy with dibutyrolactone, does not fit the ready hydrolysis of these compounds, since dibutyrolactone has been shown by Fittig and Ström [1892] on boiling with dilute mineral acid not to break down into its two C_4 compounds but to yield the extremely stable compound, oxetone:

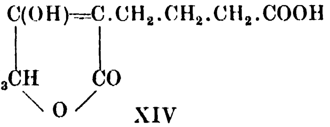
$$C_8H_{10}O_3+H_2O=C_7H_{12}O_2+CO_2.$$

(*d*) *Reduction of carolic, carolinic and α-acetyltetronic acids.*

Carolic acid, $C_9H_{10}O_4$, on reduction with palladium-charcoal-hydrogen, absorbs $2H_2$ and gives the reduced monobasic acid $C_9H_{14}O_4$ which on distillation loses H_2O and gives the neutral substance $C_9H_{12}O_3$. Carolinic acid, $C_9H_{10}O_6$, absorbs $2H_2$, giving the reduced acid $C_9H_{12}O_5$ whilst α-acetyltetronic acid $C_6H_6O_4$ also absorbs $2H_2$ at a comparable rate with carolic and carolinic acids, giving α-ethyltetronic acid. With α-acetyltetronic acid therefore the ketonic group of the side-chain is reduced to CH_2, and by analogy we might expect carolic and carolinic acids to behave similarly. Carolinic acid also gives the same general reaction, and the fact that reduced carolic acid appears to contain 1 molecule H_2O more than would be expected by analogy with reduced carolinic and α-acetyltetronic acids is explained by assuming that in reduced carolic acid the second ring becomes stabilised in the open form, and 1 molecule H_2O is lost only on distillation. All three acids before reduction give orange and after reduction red $FeCl_3$ colours, which are indistinguishable. The red colours given by the reduced acids are identical with those given by γ-methyltetronic and acetoacetic acids and are due to the keto-enol grouping in the tetronic acid ring.

Reduced carolic, carolinic and α-acetyltetronic acids (α-ethyltetronic acid) all give on hydrolysis substances which reduce Fehling's solution in the cold and slowly give bis-dinitrophenylhydrazones, and probably therefore contain the CHOH.CO grouping. This confirms the view that the tetronic acid ring itself is not affected by reduction under our conditions, and moreover γ-methyltetronic acid itself is not reduced with our catalyst.

Reduced carolic acid may therefore be represented by structure X and reduced carolinic acid by structure XIV.



XIV

It would be difficult to fit the results obtained on reduction with formula II for carolic acid and impossible with formula III for carolinic acid.

(*e*) *Absorption spectra of carolic, carolinic, α-acetyltetronic, γ-methyltetronic acids and of their reduction products.*

As will be shown in a subsequent publication, the structures assigned to the above compounds fit the requirements of their absorption spectra. These have been investigated by Dr E. L. Hirst of Birmingham University. Thus whilst carolic, carolinic and α-acetyltetronic acids all give a double-banded spectrum, one band characterising the tetronic acid ring and the second the ketone group immediately outside this ring, all the three corresponding reduced acids show only a single band corresponding to that of either γ-methyl- or α-ethyl-tetronic acid. Thus reduction results in all three cases in the loss of the ketonic absorption band.

(*f*) *Reactions of carolic, carolinic and α-acetyltetronic acids with 2:4-dinitrophenylhydrazine.*

The ketonic nature of all these compounds can be readily confirmed by means of 2:4-dinitrophenylhydrazine. Benary [1910], working with α-acetyltetronic acid and phenylhydrazine, found that a monophenylhydrazone was readily formed, and he showed that it was the carbonyl group of the side chain and not that of the tetronic acid ring which reacted. We have found that on treating α-acetyltetronic acid or carolinic acid with Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl) the yellow monodinitrophenylhydrazone is immediately precipitated. Carolic acid does not give any immediate precipitate. After standing 24–48 hours, a precipitate begins slowly to separate and continues to separate for 6–8 weeks. The precipitate initially contains a monodinitrophenylhydrazone which is fairly soluble in water, together with a bis-dinitrophenylhydrazone and after 8 weeks consists entirely of the pure bis-compound. Both compounds are dinitrophenylhydrazones of carolic acid + H_2O ($C_9H_{12}O_5$). The bis-dinitrophenylhydrazone tends to revert to the monoderivative on storing. It appears therefore that during this reaction, which is carried out in 2*N* HCl solution, H_2O is slowly added to open the second ring and that the monodinitrophenylhydrazone is then slowly precipitated and transformed into the bis-derivative.

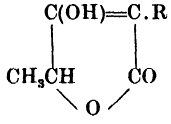
All three monodinitrophenylhydrazones are soluble and the bis-derivative of carolic acid is insoluble in bicarbonate. It would be impossible to obtain a bis-derivative with formula II for carolic acid. Attempts to obtain the bis-derivatives with α-acetyltetronic and carolinic acids, on standing 6–8 weeks with Brady's reagent, were unsuccessful. Carolinic acid still gave only the monoderivative, while α-acetyltetronic acid suffered slow hydrolytic degradation under these conditions giving the bis-dinitrophenylhydrazone of methylglyoxal. The difference in behaviour of the monodinitrophenylhydrazones of carolic and carolinic acids towards an excess of Brady's reagent is probably due to the fact that the former dinitrophenylhydrazone is very soluble whilst the latter is insoluble in the reagent.

(*g*) *Reactions of carolic, carolinic, α-acetyltetronic and γ-methyltetronic acids with diazomethane.*

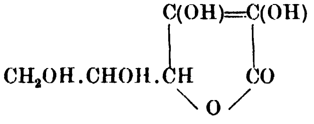
Carolic acid on treatment with diazomethane in incompletely dry ether, reacted very slowly, and after keeping overnight, removing the solvent and drying, a yellow oil was obtained which had a methoxyl content slightly greater (15·9 %) than that of a monomethyl derivative (14·5 %). This compound however slowly hydrolysed, even on shaking with water and readily on keeping overnight at room temperature with *N* NaOH, the methoxyl content of the material recovered from the alkaline solution being only 2·8 %. This behaviour was at first regarded as favouring the lactonic acid formula II for carolic acid, assuming that the lactonic ring opened to give the methyl ester. It has now been found however that the arrangement of ketonic groups in these acids renders the methoxyl group much more labile than in a normal methyl ether. Thus the methoxyl content of the monomethyl ether of γ-methyltetronic acid (I), which contains only one CO group in the β-position to the methoxyl group, fell on keeping with *N* NaOH for 24 hours from 24·5 to 14·7 %, and of the monomethyl ether of α-acetyltetronic acid (VI), which contains two CO groups in β-positions to the methoxyl group, fell from 20·7 to 3·6 %, the behaviour exactly resembling that of carolic acid itself. Carolinic acid gives a dimethyl derivative with diazomethane—a methyl ether methyl ester—which with N NaOH very readily loses one, and more slowly loses the second (presumably the ester), methyl group.

This close similarity in behaviour between α-acetyltetronic, carolic and carolinic acids, supported by the absorption spectra data, makes it very probable that in carolic and carolinic acids γ-hydroxybutyric and succinic acids are condensed respectively with γ-methyltetronic acid to give ketonic structures (IV and V) similar to α-acetyltetronic acid. These formulae have therefore been allotted to carolic ($+H_2O$) and carolinic acids respectively, which may therefore be formulated as α-[γ-hydroxybutyryl]-γ-methyltetronic and α-[β-carboxypropionyl]-γ-methyltetronic acids.

Finally the close analogy between the tetronic acid half of the molecule of carolic and carolinic acids (XV) and the structure of ascorbic acid (XVI) (vitamin C) should be noted.



XV



XVI

This analogy in structure is strongly borne out by the absorption spectra. As will be shown in a later publication, γ-methyltetronic acid gives a similar spectrum to that of ascorbic acid, and the band moves in acid and alkaline solution in the same way as does the band of ascorbic acid.

EXPERIMENTAL.

A. *General properties of carolic, carolinic and α-acetyltetronic acids.*

(i) *Carolic acid.*

Carolic acid, $C_9H_{10}O_4$, M.P. 132°, $[\alpha]_{5461}+84°$, gives a bright orange $FeCl_3$ colour and a negative nitro-chromic acid reaction for the primary alcohol grouping, but the latter test becomes positive on standing, due probably to ring opening. The immediate violet colour with $NaNO_2$, which is given by tetronic acids having the α-CH_2 group either unsubstituted or substituted by an easily removable substituent, *e.g.* bromine [Wolff, 1896], is not given immediately by carolic acid. On warming with concentrated H_2SO_4, carolic acid dissolves, giving a colourless solution which on heating to 140° becomes a deep bluish red.

An aqueous solution of the acid blues Congo red. The acid in dry anisole solution has however no active hydrogen as indicated by negative Zerewitinoff determinations by Roth's method. In pyridine, on the other hand, the acid possesses one active hydrogen. Thus 7·52 mg. carolic acid at 28° in dry anisole gave 0·02 ml. CH_4, and 9·75 mg. in pyridine gave 1·18 ml. CH_4, the latter corresponding to 0·98 active hydrogen atom. Carolic acid titrates in cold aqueous solution as a monobasic acid; thus 0·2322 g. required 12·80 ml. $N/10$ NaOH for titration to a permanent pink colour with phenolphthalein (the first pink flush was obtained with 12·6 ml. $N/10$ NaOH), corresponding to an equivalent of 181·4 ($C_9H_{10}O_4$, assumed monobasic, requires 182·1). This titration was not materially altered on heating with excess NaOH. Thus heating for ½ hour at 55°, and subsequently for a further ¾ hour at 80–85° only increased the titre from 12·8 to 13·3 and 13·7 ml. respectively.

The acid also shows a somewhat anomalous behaviour in respect to melting-point. A sample of the acid crystallised from absolute alcohol melted at 132°. Not only did the melting-point tend to fall on storage, but on melting and resetting it fell and became constant at 112°. Moreover there was no loss of water or CO_2 on heating in dry nitrogen at the melting-point although the melting-point again fell from 132 to 112°. The material melting at 112° on recrystallisation from alcohol again melted at 132°. This change may be related to the keto-enolic type of structure.

The acid gives a very soluble silver salt. Thus 0·3073 g. of carolic acid was neutralised with 17·1 ml. $N/10$ NaOH and $AgNO_3$ added. The silver salt on concentration *in vacuo* at room temperature to 5 ml. showed no signs of crystallising.

Attempted acetylation of carolic acid. Acetylation of carolic acid both by the acetic anhydride-pyridine and by the acetic anhydride-sodium acetate methods failed owing to complete decomposition and blackening. Darkening did not occur however when carolic acid was heated either with acetic anhydride or with acetyl chloride, and acetylation was therefore attempted as follows. 1 g. carolic acid was heated with 15 ml. acetyl chloride for 1 hour in a boiling water-bath, the acetyl chloride mostly distilled off and light petroleum added. The product (0·95 g.) separated as a colourless crystalline solid, was rubbed up with successive amounts of light petroleum and melted at 112°. After recrystallisation from ethyl alcohol, it melted at 132° (*cf.* previous section). The melting-point was not depressed on mixing with carolic acid. Also 0·0264 g. required 1·48 ml. $N/10$ NaOH corresponding to an equivalent of 178·3. ($C_9H_{10}O_4$, assumed monobasic, requires 182). It appears that carolic acid does not form an acetyl derivative and that it is unlikely therefore that it contains a free OH grouping.

Action of alkaline iodine on carolic acid. Although carolic acid with strong reagents (10 % NaOH and $N/10$ I) readily gives CHI_3, no CHI_3 is formed nor is iodine absorbed when the acid is titrated with alkaline iodine in very dilute solution by the method used for determination of kojic acid [Birkinshaw and Raistrick, 1931]. Thus 5 ml. of an aqueous solution containing 10·86 mg. carolic acid, on standing at room temperature for 90 minutes with 40 ml. $N/10$ iodine and 50 ml. $N/10$ NaOH showed an absorption of only 0·3 ml. $N/10$ iodine.

Action of Wijs's reagent on carolic acid. 0·3947 g. carolic acid in 10 ml. CCl_4 was treated with 25 ml. Wijs's solution. The difference in titration of the blank and the reaction fluid was only 0·8 ml. $N/10$ thiosulphate. (Theoretical for absorption of 1 molecule $I_2 = 43·4$ ml.). The absorption may therefore be regarded as negligible.

(ii) *Carolinic acid.*

Carolinic acid, $C_9H_{10}O_6$, M.P. 129° (monohydrate, M.P. 123°), $[\alpha]_{5461} + 60°$ (in water) gives with $FeCl_3$ an immediate orange precipitate soluble in excess to a bright orange coloured solution. It does not give a colour with $NaNO_2$. An aqueous solution blues Congo red. The acid titrates sharply in the cold as a dibasic acid, and determinations in dry anisole by the Zerewitinoff method for active hydrogen give values which are slightly high for two active hydrogen atoms (Roth). Thus 11·015 mg. carolinic acid gave 2·39 ml. CH_4 (at 18°) and 2·46 ml. CH_4 (at 95°). ($C_9H_{10}O_6$ requires for one active hydrogen the production of 1·05 ml. CH_4 at N.T.P.) Similar values were obtained in pyridine.

Carolinic acid like carolic acid does not react with Wijs's reagent nor does it acetylate on boiling with acetyl chloride. It does not give a bluish red colour with hot concentrated H_2SO_4 as does carolic acid.

(iii) *α-Acetyltetronic acid,* $C_6H_6O_4$.

This was synthesised by Benary's [1909] method, by condensation of ethyl β-aminocrotonate and chloroacetylchloride with subsequent hydrolysis of the condensation product. After recrystallisation from benzene it melted at 83·5°. (Found: C, 50·65; H, 4·25 %. $C_6H_6O_4$ requires C, 50·69; H, 4·26 %.) It gives an orange $FeCl_3$ colour identical with that of carolic acid but no colour with $NaNO_2$. On titration to phenolphthalein 53·1 mg. required 3·695 ml. $N/10$ NaOH corresponding to an equivalent of 143·7 ($C_6H_6O_4$ titrating as a monobasic acid requires 142).

B. *Bromination of carolic, carolinic and α-acetyltetronic acids.*

Considerable advance in our knowledge of the structure of these acids has been made by a detailed study of the products obtained by bromination under varying conditions.

(I) *Bromination of carolic acid in glacial acetic acid solution: preparation of* d-*monobromocarolic acid.* 2 g. carolic acid were dissolved in 20 ml. glacial acetic acid and a solution containing 2·5 ml. Br in 100 ml. glacial acetic acid was added 1 ml. at a time at room temperature. The reaction was slow at first but became very much more rapid, and an amount of bromine was decolorised corresponding to 2 atoms Br. Hydrobromic acid was given off. The solution was placed in a desiccator over solid KOH and the hydrobromic and acetic acids were quickly removed at room temperature at very low pressure. A thick yellow oil remained which crystallised only after standing for several weeks. The dried material from a second experiment was therefore immediately rubbed up with a little methyl alcohol and allowed to stand in the cold room, when 1·37 g. of crystals separated. The mother-liquor was boiled with a little charcoal, filtered and again placed to crystallise in the cold room, giving a second crop of crystals, weight 0·65 g. The total crude yield represents 70 % of the theory for the production of monobromocarolic acid. The crystals melted at 149–151°. Bromocarolic acid is best purified by recrystallisation (1·2 g.) from boiling methyl alcohol (14 ml.) from which solvent it separates in hard, very large homogeneous plates (0·8 g.), M.P. 158°. (Found (Schoeller): C, 41·57, 41·59; H, 3·47, 3·42; Br, 31·15, 31·29 %. $C_9H_9O_4Br$ requires C, 41·38; H, 3·48; Br, 30·61 %.)

0·1435 g. in 20 ml. absolute alcohol required 5·53 ml. $N/10$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 260 ($C_9H_9O_4Br$ titrating as a monobasic acid requires 261).

Monobromocarolic acid in water ($c = 1·06$) has $[\alpha]_{5461} = +39°$.

In a control experiment it was shown that 50 % of the bromine absorbed had been eliminated as HBr and was precipitated and estimated as AgBr.

Hydrolysis of d-*monobromocarolic acid.* This hydrolysis was carried out in order to determine whether the bromine atom had entered the butyrolactone or the methyltetronic acid half of the molecule. Thus if it had entered the methyltetronic acid ring we should expect on hydrolysis to obtain diacetyl, butyrolactone and CO_2, whereas if it had entered the butyrolactone half of the molecule we should expect acetoin, CO_2 and either bromo- or hydroxy-butyrolactone.

0·818 g. bromocarolic acid was hydrolysed by boiling for 3 hours with 20 ml. $2N$ H_2SO_4 in a stream of CO_2-free nitrogen, the gases evolved being passed up a reflux condenser and then first through a bubbler containing Brady's reagent (2 : 4-dinitrophenylhydrazine in $2N$ HCl) and finally through bubblers containing $N/10$ baryta. The baryta bubblers showed an evolution of 58·9 ml. $N/10$ CO_2 corresponding to 94 % of the theoretical for 1 molecule CO_2. The bubbler containing Brady's reagent, on standing, contained only a very slight precipitate as is usual when acetoin is formed. Diacetyl could not have been formed since this substance is so very volatile that a heavy precipitate would have been obtained under our conditions.

The hydrolysate was next cooled and titrated. On subtracting from the total titration figure the titre of the H_2SO_4 used for hydrolysis, it was found that 3·4 ml. *N* acid still remained. This corresponds closely to one equivalent of acid (theoretical 3·13 ml.) and it appears therefore that the bromo- (or hydroxy-)butyrolactone is almost entirely present as the opened-up acid. This was later confirmed by titration of the isolated acid. The neutralised solution was made up to a standard volume and aliquot portions used in the following experiments.

(*a*) A portion of the neutralised hydrolysate was distilled *in vacuo* and the distillate shown to contain acetoin by its reduction of Fehling's solution in the cold, by its positive Voges-Proskauer reaction and by the slow production, in the way characteristic of acetoin, of the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° decomp., unaltered by admixture with an authentic specimen.

(*b*) The acetoin content was determined in an aliquot by the Wood-Ost method, the reducing power of the whole hydrolysate corresponding to 0·2656 g. acetoin, representing 96 % of the theoretical from 0·818 g. of bromocarolic acid.

(*c*) The ionised Br of a portion of the hydrolysate was determined by precipitation as AgBr and corresponded to 31 % of the bromine actually present. It appears therefore that only one-third of the bromine has been separated by hydrolysis and that two-thirds still remain attached to one of the hydrolysis products, *viz.* to the butyrolactone.

(*d*) The residual neutral hydrolysate was extracted continuously with ether until acetoin was completely removed, the solution then acidified and again extracted continuously with ether. On removing the ether, a brown oil remained which was dried overnight over H_2SO_4 *in vacuo*. 0·0972 g. of this oil required, on neutralisation in the cold to phenolphthalein, 5·4 ml. *N*/10 NaOH corresponding to an equivalent of 180 ($C_4H_7O_3Br$ titrating as a monobasic acid requires 183), and contained 40·3 % Br (theoretical for $C_4H_7O_3Br = 43{\cdot}7$ %). The material did not give a crystalline phenylhydrazide as does butyrolactone under the same conditions, and on heating with NaOH lost its bromine, giving presumably a dihydroxybutyric acid which was not, however, further identified.

From these experiments it appears certain that bromination of carolic acid in glacial acetic acid leads to the absorption of 1 molecule Br and to the formation of monobromocarolic and hydrobromic acids, the former of which has its Br atom in the butyrolactone half of the molecule.

(II) *Bromination of carolic acid in aqueous solution and in 50 % acetic acid solution.* Carolic acid reacts with bromine in aqueous and in 50 % acetic acid solution in two ways:

$$\text{(i)} \qquad C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_4O_3Br_2 + C_4H_6O_2 + 2HBr$$

thus giving rise to $\alpha\gamma$-dibromo-γ-methyltetronic acid and butyrolactone;

$$\text{(ii)} \qquad C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_5O_3Br + C_4H_5O_2Br + 2HBr$$

thus forming α-bromo-γ-methyltetronic acid and bromobutyrolactone. In aqueous solution reaction (i), and in 50 % acetic acid solution reaction (ii) predominates.

Thus when 1 g. carolic acid is dissolved in 40 ml. water or 50 % acetic acid and a standardised solution of bromine in water or 50 % acetic acid respectively is added, immediate decoloration of bromine occurs and a sharp end-point is obtained after the equivalent of 2 molecules of bromine has been added. If the ionised bromine of an aliquot portion of these two solutions be then determined by precipitation as AgBr, it is found that 2 molecules of HBr have been formed in both reactions.

(*a*) *Isolation of* d-*α-bromo-γ-methyltetronic acid, $C_5H_5O_3Br$.* 1 g. of carolic acid was dissolved in 20 ml. 50 % acetic acid and the theoretical amount ($2Br_2$) of a solution containing 2·5 ml. bromine in 100 ml. 50 % acetic acid slowly added. The solution was then rapidly evaporated to dryness by standing over sticks of KOH in a high vacuum at room temperature. Under these conditions, acetic and hydrobromic acids, butyrolactone and bromobutyrolactone pass over to the KOH, together with any dibromomethyltetronic acid which may have been formed, since this is decomposed under these conditions. 0·85 g. of crude material remained (the theoretical amount for the production of 1 molecule of $C_5H_5O_3Br$ is 1·06 g.). The residual crude α-bromo-γ-methyltetronic acid was well washed with cold benzene and light petroleum and then weighed 0·60 g. M.P. 165°. After several recrystallisations from benzene it separated in beautiful long needles and the M.P. became constant at 172°. It gave a red $FeCl_3$ and a violet $NaNO_2$ colour. (Found (Schoeller): C, 31·17; H, 2·83; Br, 41·49 %. $C_5H_5O_3Br$ requires C, 31·10; H, 2·61; Br, 41·40 %.)

0·0604 g. required 6·30 ml. $N/20$ NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 192 ($C_5H_5O_3Br$ titrating as a monobasic acid requires 193).

It had in water ($c=2·7$ g.) $[\alpha]_{5461}=+9·47°$.

(*b*) *Reduction of* d-*α-bromo-γ-methyltetronic acid $C_5H_5O_3Br$ to an acid $C_5H_6O_3$, shown to be* l-*γ-methyltetronic acid.* *d*-α-Bromo-γ-methyltetronic acid (2 g.) was next reduced catalytically by palladium-charcoal-hydrogen. The sodium salt of the acid hydrogenated with great ease and was therefore used in order to minimise loss by adsorption on the charcoal.

The reduction, which was carried out as described later, was complete in about 5 minutes and corresponded to an uptake of 1 molecule H_2. The catalyst was filtered off and washed with $2N$ NaOH and then with water. The filtrate and washings were combined, acidified and extracted three times with an equal volume of ether. The ethereal extracts were dried over $MgSO_4$ and the ether removed *in vacuo*. In this way was obtained 0·71 g. of almost pure crystalline acid corresponding to 60 % of the theoretical yield, M.P. 112°. The acid recrystallised from benzene-light petroleum in curved feathery needles, M.P. 115°. It also readily sublimed *in vacuo* and the sublimate melted at 115°. (Found (Schoeller): C, 52·72, 52·75; H, 5·33, 5·30 %. $C_5H_6O_3$ requires C, 52·61; H, 5·30 %.)

0·1329 g. required 11·63 ml. $N/10$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 114 ($C_5H_6O_3$ titrating as a monobasic acid requires 114).

The free acid in water ($c=1·426$) had $[\alpha]_{5461}=-21°$.

The acid gave strong red $FeCl_3$ and violet $NaNO_2$ colours, and with Brady's reagent readily gave a crystalline monodinitrophenylhydrazone, M.P. 235°, unchanged by recrystallisation from nitrobenzene-toluene. (Found (Schoeller): C, 44·95; H, 3·31; N, 18·71 %. $C_{11}H_{10}O_6N_4$ requires C, 44·90; H, 3·43; N, 19·05 %.)

The products of hydrolysis of the acid were next investigated.

The titrated solution referred to above (0·1329 g.) was washed into a hydrolysis flask, the total volume with washings being 50 ml. 50 ml. $2N$ H_2SO_4 were added and the solution heated to boiling in a stream of CO_2-free nitrogen, the resultant gases being passed through baryta bubblers. After 3 hours' hydrolysis, 23·4 ml. $N/10$ CO_2 had been evolved (theory for 1 molecule $CO_2=23·3$ ml.). The hydrolysate was cooled, the H_2SO_4 neutralised with concentrated KOH, made up to 150 ml. and aliquot portions used for the following tests. The solution gave a positive Voges-Proskauer reaction, reduced Fehling's solution in the cold, slowly in the manner characteristic of acetoin gave the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (not depressed by mixing with an authentic specimen), and on determination of the acetoin content by the Wood-Ost method was shown to contain 0·1004 g. of carbinol (theory for 1 molecule acetoin 0·1026 g. carbinol).

The acid melted at 115°, was laevorotatory, titrated as a monobasic acid, contained one CO group and gave on hydrolysis 1 molecule each of CO_2 and acetoin. It seemed certain therefore that it was *l*-γ-methyltetronic acid the *dl*-form of which had previously been synthesised by Benary [1911], who gives the M.P. as 117°. The *dl*-variety of γ-methyltetronic acid was accordingly synthesised by Benary's method by condensation of ethyl sodiomalonate with chloroacetyl chloride and subsequent hydrolytic decarboxylation. It melted at 115°. The synthetic acid gave the same red $FeCl_3$ and violet $NaNO_2$ colours, and on hydrolysis under the conditions quoted above gave quantitatively 1 molecule each of CO_2 and acetoin. Although the melting-points of the two samples of methyltetronic acid were the same, they showed a slight depression on mixing due, undoubtedly, to the fact that the synthetic acid was the *dl*-variety, whereas the acid from the monobromide was the *l*-variety. Both samples of acid, however, gave the same dinitrophenylhydrazone, M.P. 235°, and the two hydrazones on admixture showed no depression of melting-point.

The acid was brominated in dry chloroform under conditions used by Wolff [1896] for bromination of tetronic acid, *i.e.* conditions leading with certainty to the formation of *d*-α-bromo-γ-methyltetronic acid. An acid was thus readily obtained which after recrystallisation from benzene had M.P. 172°. The acid gave a red $FeCl_3$ and violet $NaNO_2$ colour and showed no depression of M.P. on mixing with the *d*-α-bromo-γ-methyltetronic acid, $C_5H_5O_3Br$, obtained by bromination of carolic acid in 50 % acetic acid solution. It is certain therefore that the acid split off from carolic acid on bromination in 50 % acetic acid solution is *d*-α-bromo-γ-methyltetronic acid.

(*c*) *Isolation of the dibromide* $C_5H_4O_3Br_2$ *and its identification as* d-$\alpha\gamma$-*dibromo*-γ-*methyltetronic acid.* 1 g. of carolic acid was dissolved in 40 ml. water and the theoretical amount of bromine water added. Using the silver method it was shown that 50 % of the bromine absorbed was eliminated as HBr. The solution was then extracted three times with an equal volume of ether, the ethereal extracts dried with anhydrous $MgSO_4$, filtered and the ether removed *in vacuo*. In this way 1·55 g. of a product were obtained consisting chiefly of beautifully crystalline material together with a little oil, presumably butyrolactone. The product was dissolved in dry ether and two volumes of light petroleum were added. On standing 0·38 g. of large crystals, consisting of a mixture of long colourless prisms and flat hard plates was obtained, M.P. 114–5°. It was found impracticable to separate this mixture by fractional crystallisation but a satisfactory separation was effected by fractional sublimation into the anhydrous dibromide $C_5H_4O_3Br_2$, prisms, M.P. 88°, and its monohydrate $C_5H_4O_3Br_2, H_2O$, plates, M.P. 135°. The hydrate loses water at the melting-point and, after resetting, melts constantly at 88°. (Found for anhydrous substance (Schoeller): C, 21·82; H, 1·48 %. $C_5H_4O_3Br_2$ requires C, 22·08; H, 1·49 %: Found for hydrate: C, 20·90; H, 2·05 %. $C_5H_4O_3Br_2$, H_2O requires C, 20·70; H, 2·09 %.)

Bromine determinations on these two compounds were unsatisfactory, probably owing to the large amount of bromine in the molecule. Thus a duplicate specimen of each of the anhydrous and hydrated materials was submitted to two independent analysts (*a*) and (*b*) with the following results: *Anhydrous dibromide*, Found: (*a*) 57·4 %; (*b*) 55·9 %. ($C_5H_4O_3Br_2$ requires 58·8 %.) *Monohydrate*, Found: (*a*) 53·4 %; (*b*) 52·7 %. ($C_5H_4O_3Br_2$, H_2O requires 55·1 %.)

Specific rotation of monohydrate in water ($c = 2{\cdot}04$) $[\alpha]_{5461} = +36{\cdot}2°$.

Both dibromides on titration to an initial end-point, followed by keeping overnight at room temperature with excess $N/10$ NaOH, showed the presence of one acidic and one lactonic grouping, from which it follows that the bromide M.P. 135°, $C_5H_4O_3Br, H_2O$ is a true hydrate and not the opened-up acid. Accurate titration values could not be obtained owing to the partial hydrolysis (about 8 %) of the bromine atoms on standing with $N/10$ NaOH at room temperature overnight.

Both the hydrated and anhydrous forms of the dibromide on hydrogenation with Pd-charcoal and hydrogen readily give a monobasic acid, $C_5H_6O_3$, M.P. 115°, giving a red $FeCl_3$ colour and a violet $NaNO_2$ colour, which shows no depression of melting-point when mixed with the corresponding acid obtained by hydrogenation of *d*-α-bromo-γ-methyltetronic acid. This acid $C_5H_6O_3$ has been shown to be *l*-γ-methyltetronic acid. The dibromide is therefore almost certainly αγ-dibromo-γ-methyltetronic acid, since with this formulation the substance would behave as a mono-acid monolactone, and moreover, as we have shown, αα′-dibromo-γ-methyltetronic acid, like αα′-dibromotetronic acid [Wolff, 1896] is a very unstable neutral substance and in presence of water readily decomposes.

(III) *Bromination of carolic acid first in glacial acetic acid, followed by dilution to 50 °/₀ acetic acid and further bromination.* The best yields of *d*-α-bromo-γ-methyltetronic acid were obtained by adding bromine (1 molecule) in glacial acetic acid solution to a solution of carolic acid (1 molecule) in the same solvent, diluting to 50 % acetic acid solution and then adding a second molecule of bromine in 50 % acetic acid. The bromide was then isolated as in the previous experiments and the yield proved to be almost theoretical. The bromide did not depress the M.P. of the specimen prepared as previously described.

(IV) *Bromination of carolinic acid in 50 °/₀ acetic acid solution.* Carolinic acid (1 g.) was brominated in 50 % acetic acid solution exactly as in the experiment with carolic acid; 2 molecules bromine were absorbed giving rise as was shown by precipitation as AgBr to 2 molecules HBr. The solution was evaporated in high vacuum over KOH to dryness. A crystalline residue remained which was extracted with boiling benzene. The insoluble portion was filtered off and the filtrate, on cooling, deposited crystals of *d*-α-bromo-γ-methyltetronic acid which were shown to be identical with the bromide from carolic acid. On bromination, carolinic acid gives, therefore, the same *d*-α-bromo-γ-methyltetronic acid as does carolic acid.

The benzene-insoluble residue was carefully sublimed and the sublimate resublimed. A white powder was obtained which melted at 188° and did not depress the M.P. of an authentic specimen of succinic acid. On titration to phenolphthalein, 0·0641 g. required 10·80 ml. $N/10$ NaOH corresponding to an equivalent of 59·4 ($C_4H_6O_4$ titrating as a dibasic acid requires 59).

(V) *Bromination of carolinic acid in aqueous solution.* 1 g. of carolinic acid was brominated in aqueous solution exactly as with carolic acid, in order to discover whether the same αγ-dibromo-γ-methyltetronic acid was also produced. Here on evaporation to dryness a crystalline residue remained which was shown to consist of this dibromide and succinic acid. With carolic acid the products were the dibromide and the volatile oily butyrolactone and these were readily separated. Greater difficulty was experienced with the products from carolinic acid. The readiest separation was obtained by fractional sublimation. On subliming, succinic acid first formed the usual compact sublimate whilst below this the dibromide formed very characteristic large well-shaped prisms. These were easily detached and after resublimation had M.P. 88°, not depressed by mixing with a sample of the dibromide $C_5H_4O_3Br_2$ from carolic acid. The succinic acid was also resublimed and characterised by M.P., mixed M.P. with an authentic specimen, and by titration.

It appears, therefore, that carolinic acid on bromination gives the same *d*-α-bromo- and *d*-αγ-dibromo-γ-methyltetronic acids as does carolic acid, and this was confirmed by reduction of the two bromides, when the same *l*-γ-methyltetronic acid resulted, which gave the same monodinitrophenylhydrazone as was obtained in the experiments with carolic acid.

(VI) *Bromination of α-acetyltetronic acid.* 1·19 g. of α-acetyltetronic acid were dissolved in 15 ml. 50 % acetic acid and a standard solution of bromine in 50 % acetic acid was added from a burette. The solution, which absorbed 2 molecules bromine, was evaporated as before over KOH in a high vacuum and gave a colourless crystalline solid. After recrystallisation from benzene it melted at 183°. (Found: C, 26·95; H, 1·94; Br, 44·30 %. $C_4H_3O_3Br$ requires C, 26·83; H, 1·69; Br, 44·66 %.) It did not depress the M.P. of a synthetic sample of α-bromotetronic acid [Wolff, 1896]. Like the latter, it gave a violet colour with $NaNO_2$, bromine being displaced from the α-position. These facts show conclusively that during the bromination of α-acetyltetronic acid, the acetyl group is removed and replaced by a bromine atom in the same way as are the side chains of carolic and carolinic acids.

C. *Hydrolysis of carolic, carolinic and α-acetyltetronic acids.*

One of the most characteristic properties of these acids is their very ready hydrolysis by boiling dilute acids and alkalis.

(I) *Hydrolysis of carolic acid.* Carolic acid (5 g.) was hydrolysed by boiling under a reflux condenser for 3 hours with 250 ml. $2N H_2SO_4$ in a current of CO_2-free nitrogen, the gases evolved being passed through bubblers containing standard baryta. CO_2 equivalent to 524 ml. $N/10$ baryta was produced. The evolution of 1 molecule CO_2 by 1 molecule of carolic acid requires 549 ml. baryta.

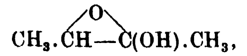
The hot hydrolysis solution was neutralised with N NaOH to a permanent end-point, the solution titrating in the manner of a lactone. The neutral solution was then distilled *in vacuo* to dryness several times and the distillate and residue were examined separately.

(*a*) *Distillate.* This was made up to 1250 ml. and aliquot portions were examined.

(1) A portion readily reduced Fehling's solution in the cold and gave a strong Voges-Proskauer reaction usually regarded as characteristic of acetoin and diacetyl.

(2) On adding excess 2 : 4-dinitrophenylhydrazine in $2N$ HCl, an orange turbidity was produced and a crystalline bis-dinitrophenylhydrazone slowly separated on standing 6 weeks, which after recrystallisation from nitrobenzene-toluene melted at 318°. Samples of diacetyl and acetoin were similarly treated with Brady's reagent. Diacetyl immediately gave a heavy precipitate of the yellow monodinitrophenylhydrazone which on standing overnight with excess of reagent was gradually converted into the orange bis-dinitrophenylhydrazone. This, on crystallisation from nitrobenzene-toluene, melted at 318°. With acetoin, however, an orange turbidity was first obtained and an orange bis-dinitrophenylhydrazone, M.P. 318°, gradually separated over a period of 6 weeks. It did not depress the melting-point of the bis-dinitrophenylhydrazone of diacetyl.

Acetoin, therefore, which according to Kling [1905, 1, 2; 1906] is not a hydroxyketone but has the structure



$$CH_3.CH\overset{O}{—}C(OH).CH_3,$$

does not give an immediate precipitate with 2:4-dinitrophenylhydrazine, but is slowly oxidised to diacetyl.

The bis-dinitrophenylhydrazone obtained from the distillate gave the following figures on micro-analysis (Schoeller): C, 43·44, 43·35; H, 3·25, 3·28; N, 24·99, 24·87 %. $C_{16}H_{14}O_8N_8$ requires C, 43·04; H, 3·16; N, 25·11 %.

It did not depress the melting-point of the bis-dinitrophenylhydrazone of diacetyl, but the manner of its separation showed conclusively that acetoin and not diacetyl was the actual product of hydrolysis.

(3) The amount of acetoin present was estimated by the reducing power as determined by the Wood-Ost method. 10 ml. contained 0·016 g. acetoin. Hence the total reducing power corresponded to that of 2 g. of the carbinol (theory 2·42 g. assuming that 1 molecule carolic acid gives 1 molecule acetoin). As a trace of acetoin is lost in the stream of nitrogen during hydrolysis this result may be regarded as sufficiently accurate.

(4) The amount of acetoin present was also estimated by weighing the bis-dinitrophenylhydrazone. 250 ml. with excess of Brady's reagent gave, after standing 6 weeks, 2·39 g. dinitrophenylhydrazone. The theoretical amount of hydrazone assuming $C_9H_{10}O_4 \rightarrow C_{16}H_{14}O_8N_8$ is 2·45 g.

(*b*) *Residue.* The residue was dissolved in a little water, acidified with 2*N* H_2SO_4 and the solution exhaustively extracted with ether. After removal of the ether, a very mobile sweet-smelling light brown oil remained and was dried for a short time *in vacuo* over H_2SO_4. This was shown to be butyrolactone as follows.

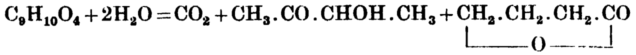
On distillation at the ordinary pressure, it almost completely passed over at 203°. It was redistilled and a sample on micro-analysis gave the following figures (Schoeller): C, 55·48, 55·58; H, 7·03, 7·06 %. $C_4H_6O_2$ requires C, 55·77; H, 7·03 %. 0·0930 g. required on titration in the cold less than 0·1 ml. *N*/10 NaOH, and after heating for ½ hour with excess NaOH and back-titrating, 10·75 ml. corresponding to an equivalent of 86·5 ($C_4H_6O_2$ titrating as a monobasic acid requires 86).

The oil on oxidation with HNO_3 (1:1) readily gave succinic acid. On heating with a slight excess of phenylhydrazine in a few ml. alcohol for 2 hours on a boiling water-bath, it gave a phenylhydrazide which crystallised in large shining plates from ethyl acetate, M.P. 94°. (Found: C, 61·88, 61·87; H, 7·09, 7·26; N, 14·68, 14·45 %. $C_{10}H_{14}O_2N_2$ requires C, 61·83; H, 7·27; N, 14·43 %.)

The phenylhydrazide did not depress the melting-point of a specimen prepared from butyrolactone which had been synthesised by the method of Fittig and Chanlaroff [1884]. Similar production of a hydrazide by valerolactone was early recorded by Wislicenus [1887].

The amount of butyrolactone produced on hydrolysis was estimated in a separate experiment as follows. 2·1521 g. carolic acid were hydrolysed as above, washed into a 700 ml. Erlenmeyer flask and titrated with *N* NaOH, first in the cold to neutralise the mineral acid present, and then after heating to estimate the butyrolactone. The total amount of *N* NaOH used by the butyrolactone was 11·90 ml. Assuming that 1 molecule carolic acid gives 1 molecule butyrolactone and no other acid products, 11·82 ml. *N* alkali would be required.

It is thus evident that hydrolysis of carolic acid gives rise to CO_2, acetoin and butyrolactone according to the equation



$$C_9H_{10}O_4+2H_2O=CO_2+CH_3.CO.CHOH.CH_3+\underbrace{CH_2.CH_2.CH_2.CO}_{O}$$

(II) *Hydrolysis of carolinic acid.* 1·004 g. carolinic acid (the hydrated form $C_9H_{10}O_6$, H_2O, M.P. 122° was used) were hydrolysed by boiling with 40 ml. dilute H_2SO_4 (equivalent to 95·2 ml. *N* acid) in a stream of CO_2-free nitrogen, the gases evolved being passed through standard baryta as with carolic acid. After 3 hours the equivalent of 84·0, after 4 hours of 85·9 ml. *N*/10 alkali had been used. The reaction $C_9H_{10}O_6, H_2O \rightarrow CO_2$ requires the production of 86·5 ml. *N*/10 CO_2. 1 molecule of CO_2 had therefore been produced.

The residual fluid and washings after cooling required 103·8 ml. *N* alkali for neutralisation in the cold, corresponding to a residual acidity of 103·8 − 95·2 = 8·6 ml. *N* acid. This figure was not increased on heating with excess NaOH. Assuming that a dibasic acid remained after hydrolysis, the residual acidity should be equivalent to 8·65 ml. *N* acid. The neutralised fluid was then evaporated *in vacuo* to dryness, water added and the process repeated five times. The combined distillates were made up to a known volume. This solution reduced Fehling's solution readily in the cold, gave a positive Voges-Proskauer reaction, and a portion of it with dinitrophenylhydrazine in 2*N* HCl slowly, after the manner of acetoin, gave the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°, the M.P. not being depressed by admixture with an authentic specimen. The amount of acetoin in an aliquot portion of the distillate was determined as with carolic acid by the Wood-Ost method. The solution contained 0·3639 g. carbinol. The reaction $C_9H_{12}O_7 \rightarrow C_4H_8O_2$ requires from 1·004 g. carolinic acid, the production of 0·3808 g. carbinol. The amount recovered represents therefore 96 % of the theoretical for 1 molecule acetoin.

The residue remaining after distillation was dissolved in a little water, acidified and extracted continuously with ether. After removal of ether and drying, 0·528 g. of very pale brown crystalline material remained, which was shown to be succinic acid. The theoretical amount of succinic acid assuming that 1 molecule carolinic acid gave 1 molecule succinic acid is 0·51 g.

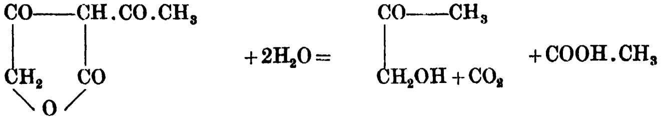
Carolinic acid therefore is hydrolysed with the same ease as carolic acid, giving 1 molecule each of CO_2, acetoin and succinic acid, according to the equation:

$$C_9H_{10}O_6+2H_2O=CO_2+CH_3.CO.CHOH.CH_3+COOH.CH_2.CH_2.COOH.$$

(III) *Hydrolysis of α-acetyltetronic acid.* 1·0551 g. of the acid were dissolved in 40 ml. dilute H_2SO_4 (≡ 82·6 ml. *N* H_2SO_4) and hydrolysed as with carolic acid. Immediately the fluid began to boil, CO_2 was evolved rapidly, as with carolic, carolinic and γ-methyltetronic acids. The total amount of CO_2 evolved corresponded to 146 ml. *N*/10 baryta. (The theoretical amount, assuming that 1 molecule $C_6H_6O_4$ gives 1 molecule CO_2 is 148·6 ml.) The hydrolysis fluid required 90·0 ml. *N* NaOH for neutralisation and there was, therefore, in addition to mineral acid 7·4 ml. *N* acid remaining after hydrolysis. Assuming that 1 molecule of a monobasic acid had been formed, the residual acidity would correspond to 7·43 ml. *N* acid. The neutralised fluid was distilled to dryness several times *in vacuo* and the combined distillates made up to a known volume. This liquid immediately reduced Fehling's solution in the cold, and with 2:4-dinitrophenylhydrazine gave slowly, over a period of 6 weeks, after the manner of acetoin, an orange-red precipitate which after recrystallisation from nitrobenzene-toluene melted at 304°. It did not depress the M.P. of an authentic specimen of the bis-dinitrophenylhydrazone of methylglyoxal. From the slow formation, oxidation appeared to occur and the actual hydrolysis product was very probably acetol. (Found: C, 41·78; H, 2·97; N, 25·97 %. $C_{15}H_{12}O_8N_8$ requires C, 41·66; H, 2·80; N, 25·92 %.)

The residual salts, on treatment with *p*-phenylphenacyl bromide [Drake and Bronitsky, 1930], gave an ester of M.P. 111°, which was not depressed by mixing with an authentic specimen of the phenylphenacyl ester of acetic acid. (Found: C, 75·67; H, 5·53 %. $C_{16}H_{14}O_3$ requires C, 75·56; H, 5·55 %.)

α-Acetyltetronic acid therefore breaks down on acid hydrolysis in the same way and with the same ease as do carolic and carolinic acids, giving 1 molecule each of acetol, CO_2 and acetic acid.



D. *Reduction of carolic, carolinic and α-acetyltetronic acids.*

In these and all subsequent reductions a palladium-charcoal catalyst was used. This was prepared as follows: 0·2 g. palladium chloride was dissolved in 25 ml. 2*N* HCl, shaken with 1 g. norite and saturated with hydrogen. The catalyst was filtered off and washed thoroughly with water. The activity of the catalyst appeared to be even greater after such short exposure to air, agreeing with the results of Willstätter and Waldschmidt-Leitz [1921]. The catalyst was now returned to the shaking apparatus and resaturated with hydrogen. The substance to be reduced was dissolved in the appropriate solvent, added to the catalyst and shaken, the rate of absorption of hydrogen being measured.

(I) *Reduction of carolic and carolinic acids.* With carolic acid (1 g. in 50 ml. water) and carolinic acid (1 g. in 50 ml. alcohol) the reduction was complete in 3 hours and hydrogen corresponding to $2H_2$ was absorbed in each case.

With carolic acid the reduction product, after filtering off the catalyst, was isolated by continuous extraction with ether, the ethereal extract being dried with $MgSO_4$ and the solvent removed. A colourless oil remained which did not crystallise even on long standing. It only became constant in weight when dried in high vacuum at 60–70° for 10 hours. (Found: C, 58·11, 58·31; H, 7·85, 7·81 %. $C_9H_{14}O_4$ requires C, 58·03; H, 7·58 %.)

0·1736 g. dissolved in water required 9·50 ml. *N*/10 NaOH for neutralisation in the cold to phenolphthalein corresponding to an equivalent of 183 ($C_9H_{14}O_4$ titrating as a monobasic acid requires 186). It appears therefore that in the reduction of carolic acid, $C_9H_{10}O_4$, $2H_2$ are absorbed and the reduced acid $C_9H_{14}O_4$ contains $2H_2$ more than carolic acid.

The reduced acid, on distillation in a high vacuum, loses 1 molecule H_2O, giving a neutral product which after recrystallisation from ether-light petroleum melted at 45°. (Found (Schoeller): C, 64·19, 64·10; H, 7·11, 7·19 %. $C_9H_{12}O_3$ requires C, 64·24; H, 7·19 %.) This compound does not titrate in the cold with dilute NaOH.

These results are explained with our formula for carolic acid (VIII) by assuming that on dissolving carolic acid in water before reduction, 1 molecule H_2O is added, thus opening the 7-membered ring (IV), that the CO group of the side chain so formed is reduced to CH_2 and the molecule becomes stabilised in the open form (X), but that on distillation 1 molecule H_2O is again split off with reformation of the 7-membered ring (XI).

With carolinic acid, the reduction product was isolated, after filtering off the catalyst, by evaporating the alcoholic fluid *in vacuo* to a small volume, when the reduced acid crystallised. After recrystallising from water it melted at 143°. (Found: C, 53·95, 54·15; H, 6·05, 6·05 %. $C_9H_{12}O_5$ requires C, 53·98; H, 6·05 %.)

0·0703 g. required 6·95 ml. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 101 ($C_9H_{12}O_5$ titrating as a dibasic acid requires 100). It appears therefore that in the reduction of carolinic acid, $C_9H_{10}O_6$, $2H_2$ are absorbed and that the product has the empirical formula $C_9H_{12}O_5$. This is readily explained by assuming that the CO group immediately outside the tetronic acid ring in (V) is reduced to CH_2.

(II) *Reduction of α-acetyltetronic acid.* 0·7 g. of acetyltetronic acid was dissolved in 25 ml. of water and reduced with palladium-charcoal-hydrogen as with carolic acid. 2 molecules of hydrogen were absorbed. The reduction was complete in about 3 hours, the rate being identical with those for carolic and carolinic acids. The reduced acid, after filtering off the catalyst, was recovered by continuous ethereal extraction. On removing the ether a colourless crystalline residue was obtained which after recrystallisation from benzene melted at 127–28°. (Found: C, 56·14; H, 6·28 %. $C_6H_8O_3$ requires C, 56·22; H, 6·30 %.) The acid did not depress the M.P. of a sample of α-ethyltetronic acid which was synthesised by bromination of ethyl α-ethylacetoacetate in ether with subsequent ring closure by removal of ethyl bromide on heating [Wedel, 1883]. The reduction reaction is therefore $C_6H_6O_4+2H_2=C_6H_8O_3+H_2O$, and the product is undoubtedly α-ethyltetronic acid having the CO group immediately outside the tetronic acid ring in (VI) reduced to CH_2.

The close similarity in structure between the three acids is further seen from the fact that whereas carolic, carolinic and α-acetyltetronic acids give orange $FeCl_3$ colours, the corresponding reduced acids all give red $FeCl_3$ colours indistinguishable from those of γ-methyltetronic or acetoacetic acids. None of them gives a colour with $NaNO_2$.

(E) *Hydrolysis of reduced carolic and carolinic acids.*

(*a*) *Reduced carolic acid.* 1·079 g. of dried reduced carolic acid were hydrolysed by boiling with 50 ml. dilute H_2SO_4 in a stream of CO_2-free nitrogen, the CO_2 evolved being absorbed in the usual way in baryta bubblers. The hydrolysis was effected very much more slowly than with carolic acid itself, hydrolysis of which was complete in less than 3 hours. Thus the amounts of CO_2 evolved in 7, 15, 26, 34 and 41 hours' boiling respectively corresponded to 42·1, 72·6, 90·8, 98·3 and 101·3 ml. *N*/10 baryta. The theoretical amount of CO_2, assuming that 1 molecule $C_9H_{14}O_4$ gives 1 molecule CO_2, corresponds to 116 ml. *N*/10 baryta. The hydrolysis was stopped after 42 hours, the flask cooled and the solution titrated. Whereas the H_2SO_4 originally used required 102·2 ml. *N* NaOH for neutralisation, the hydrolysis fluid required 102·8 ml. *N* NaOH. It appears therefore, since hydrolysis was not absolutely complete, that no acid other than CO_2 had been formed during hydrolysis.

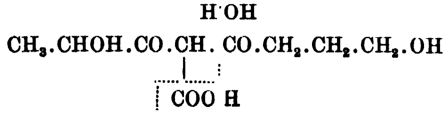
The neutralised solution was made up to 250 ml. It no longer gave any $FeCl_3$ colour. It reduced Fehling's solution readily in the cold, and on distillation of an aliquot portion to dryness *in vacuo* at 45° the substance causing reduction of Fehling's solution did not pass over into the distillate but could be extracted by means of ether from the salts remaining. The reducing substance was not therefore acetoin but a neutral non-volatile substance. The solution gave with 2:4-dinitrophenylhydrazine in 2 *N* HCl an immediate orange turbidity, and a crystalline product slowly separated during 2 weeks which after recrystallisation from nitrobenzene-toluene melted at 225° and gave the following figures on micro-analysis: C, 46·72, 46·69; H, 4·31, 4·47; N, 21·39, 21·58 %. $C_{20}H_{22}O_9N_8$ requires C, 46·31; H, 4·28; N, 21·61 %. This is obviously the bis-dinitrophenylhydrazone of a diketone $C_8H_{14}O_3$ and, since from the slow formation of the dinitrophenylhydrazone oxidation has occurred, as previously noted with acetoin, the product of hydrolysis is most probably the corresponding ketone-alcohol $C_8H_{16}O_3$. Such a compound would arise from reduced carolic acid by hydrolytic decarboxylation. $C_9H_{14}O_4 + H_2O = C_8H_{16}O_3 + CO_2$.

Ethereal extraction of the residual salts gave a colourless oil which did not crystallise even on long standing. No method was found of isolating this ketone-alcohol in a state of complete purity. On distillation in a high vacuum condensation occurred, a change which we have frequently encountered with other ketone-alcohols.

(*b*) *Reduced carolinic acid.* The hydrolysis was carried out as before. 1·1896 g. reduced carolinic acid gave amounts of CO_2 corresponding to 73·0, 105·3, 109·9, 113·9 ml. *N*/10 baryta in 6, 20, 27 and 34 hours respectively. The theoretical amount of CO_2, assuming that 1 molecule $C_9H_{12}O_5$ gives 1 molecule CO_2 is 119 ml. *N*/10 baryta. In this experiment, unlike that with reduced carolic acid, an amount of residual acid resulted which corresponded to 6·5 ml. *N* acid. Assuming that 1 molecule of a monobasic acid had been formed, the residual acid should correspond to 5·95 ml. *N* acid. The neutral solution was then evaporated to a small volume, acidified with H_2SO_4 and extracted continuously with ether. After removal of ether and drying, a pale yellow oil remained which did not crystallise and could not be obtained in a state of purity. On neutralisation in the cold to phenolphthalein, 92·2 mg. of this oil required 5·12 ml. *N*/10 NaOH corresponding to an equivalent of 180 ($C_8H_{14}O_4$ requires 174). The oil reduced Fehling's solution readily in the cold and was characterised by the fact that with dinitrophenylhydrazine in 2*N* HCl it slowly gave, in the manner of acetoin, a bis-dinitrophenylhydrazone, which after recrystallisation from nitrobenzene-toluene melted at 248° and was soluble in sodium bicarbonate. (Found: C, 45·34; H, 3·93; N, 20·23 %. $C_{20}H_{20}O_{10}N_8$ requires C, 45·09; H, 3·79; N, 21·04 %.) This would correspond to a bis-dinitrophenylhydrazone of a diketone $C_8H_{12}O_4$ and since its formation was slow and oxidation probably occurred as with acetoin the hydrolysis product was probably $C_8H_{14}O_4$ as required by the equation $C_9H_{12}O_5 + H_2O = C_8H_{14}O_4 + CO_2$.

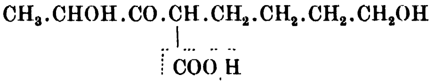
It has been shown that acid hydrolysis of reduced carolic and carolinic acids proceeds much more slowly than that of the parent acids. Further, while the parent acids each give rise to two C_4 compounds on hydrolysis—acetoin and butyrolactone in the case of carolic acid and acetoin and succinic acid from carolinic acid—each of the two reduced acids gives rise to a C_8 hydroxyketone. The results also indicate that the tetronic acid ring in carolic and carolinic acids has not been attacked during reduction, a conclusion which is supported by the fact that γ-methyl- and α-ethyl-tetronic acids are not reduced under the conditions of our reduction experiment.

These facts are readily explained from the formulae proposed for carolic acid (IV) and carolinic acid (V). Writing these structural formulae with the tetronic acid ring opened, as occurs during hydrolysis, we find

(1) 

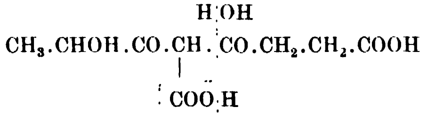
$$\mathrm{CH_3.CHOH.CO.\underset{COO\,H}{\overset{H\,OH}{CH}}.\,CO.CH_2.CH_2.CH_2.OH}$$

Carolic acid and its hydrolysis products; acetoin, CO_2 and γ-hydroxybutyric acid (butyrolactone).

(2) 

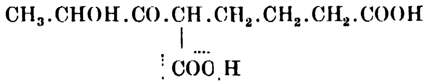
$$\mathrm{CH_3.CHOH.CO.\underset{\overset{|}{COO.H}}{CH}.CH_2.CH_2.CH_2OH}$$

Reduced carolic acid and its hydrolysis product $C_8H_{16}O_3$, *i.e.* octan-6-one-1:7-diol.

(3) 

$$\mathrm{CH_3.CHOH.CO.\underset{\overset{|}{COO.H}}{CH}.\overset{H:OH}{\vdots}CO.CH_2.CH_2.COOH}$$

Carolinic acid and its hydrolysis products, acetoin, CO_2 and succinic acid.

(4) 

$$\mathrm{CH_3.CHOH.CO.\underset{\overset{|}{COO.H}}{CH}.CH_2.CH_2.CH_2.COOH}$$

Reduced carolinic acid and its hydrolysis product $C_8H_{14}O_4$, *i.e.* ζ-hydroxy-ϵ-keto-*n*-octanoic acid.

F. *Reactions of carolic, carolinic and α-acetyltetronic acids with 2:4-dinitrophenylhydrazine.*

(I) *Carolic acid.* Carolic acid does not give an immediate precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl) but, on standing with excess of reagent, a voluminous crystalline precipitate begins to separate after 48 hours and continues to increase over a period of several weeks. Thus, using 1·0024 g. carolic acid and a large excess of reagent, the dry weight of hydrazone was after 1 week 1·70 g.; after 2 weeks 2·06 g.; after 3 weeks 2·45 g.; and after 8 weeks 3·12 g. (Theory, assuming total conversion into $C_{21}H_{20}O_{11}N_8$, 3·08 g.) This material appeared homogeneous and melted at 219°. After repeated crystallisation from nitrobenzene-toluene it separated in orange needles melting at 225° (decomp.). A sample was dried and analysed. (Found (Schoeller): C, 45·02, 45·15; H, 3·61, 3·66; N, 20·03, 20·10 %. $C_{21}H_{20}O_{11}N_8$ requires C, 44·98; H, 3·60; N, 19·99 %.) The substance is insoluble in bicarbonate and is probably the bis-dinitrophenylhydrazone of the opened-up form of carolic acid $C_9H_{12}O_5$ (IV).

When the above reaction was stopped after 8–10 days, the product contained in addition to the above bis-dinitrophenylhydrazone, the monodinitrophenylhydrazone of carolic acid. This was separated from the bis-dinitrophenylhydrazone by fractional recrystallisation from absolute alcohol in yellow needles melting at 176° (decomp.). (Found (Schoeller): C, 47·23; H, 4·19; N, 14·82 %. $C_{15}H_{16}O_8N_4$ requires C, 47·36; H, 4·24; N, 14·73 %.)

The substance is readily soluble in bicarbonate and is therefore the monodinitrophenylhydrazone of the opened-up form of carolic acid $C_9H_{12}O_5$.

It is fairly soluble in water and this probably explains the fact that the hydrazone does not appear immediately on treating carolic acid with Brady's reagent. The slow formation of these precipitates may also be due to the slow opening in 2*N* HCl solution of the 7-membered ring of carolic acid, with the slow unmasking of the second carbonyl group.

The same products can be obtained more quickly by allowing carolic acid and dinitrophenylhydrazine to react in alcoholic solution, as in Brady's more recent method [1931].

(II) *Carolinic acid.* Carolinic acid gives an immediate yellow precipitate with dinitrophenylhydrazine in 2*N* HCl. This material after recrystallisation from nitrobenzene-toluene separated in orange needles melting at 228°. A sample was dried and analysed. (Found (Schoeller): C, 46·06, 46·06; H, 3·81, 3·78; N, 14·14, 14·19 %. $C_{15}H_{14}O_9N_4$ requires C, 45·67; H, 3·58; N, 14·21 %.) The substance is soluble in bicarbonate and is therefore the monodinitrophenylhydrazone of carolinic acid.

When this derivative was allowed to stand for 8 weeks in contact with a large excess of Brady's reagent there was no further reaction.

(III) *α-Acetyltetronic acid.* According to Benary [1910] this acid with phenylhydrazine gives a monophenylhydrazone, the CO group of the side chain, and not of the tetronic acid ring, reacting. With 2:4-dinitrophenylhydrazine, also, it gives an immediate yellow precipitate, which after recrystallisation from nitrobenzene-toluene was obtained as yellow needles, melting at 226°, and was soluble in $NaHCO_3$. (Found (Schoeller): C, 44·92, 44·81; H, 3·19, 3·25; N, 17·17, 17·15 %. $C_{12}H_{10}O_7N_4$ requires C, 44·71; H, 3·13; N, 17·39 %.)

It was found however that if this monodinitrophenylhydrazone were kept with excess of the reagent for 6–8 weeks, it was slowly degraded by acid hydrolysis, giving the bis-dinitrophenylhydrazone of methylglyoxal.

G. *Reactions of carolic, carolinic, γ-methyltetronic and α-acetyltetronic acids with diazomethane.*

The acids were suspended in commercial dry (incompletely dry) ether and a solution of diazomethane in ether was added. With carolinic, γ-methyltetronic and α-acetyltetronic acids the reaction was vigorous, but with carolic acid it proceeded slowly for several hours. After standing overnight with excess of reagent, the ethereal solutions were evaporated and dried over H_2SO_4 *in vacuo* for several days. In all cases yellow oils resulted. With γ-methyltetronic acid, the oil contained 24·5 % OMe ($C_5H_5O_2OMe$ requires 24·2 % OMe), with carolic acid 15·9 % OMe ($C_9H_9O_3OMe+H_2O$ requires 14·5 % OMe), with carolinic acid 28·4 % OMe ($C_9H_8O_4(OMe)_2$ requires 25·6 % OMe), and with α-acetyltetronic acid 20·7 % OMe ($C_6H_5O_3OMe$ requires 19·9 % OMe).

Valuable information as to the nature of these methyl derivatives, particularly in relation to the molecular constitutions of carolic and carolinic acids, was obtained by examining their behaviour towards cold dilute NaOH.

The four methylation products (1 g. in each case) were kept at room temperature for 24 hours with 20 ml. *N*/NaOH.

The monomethyl derivative of γ-methyltetronic acid almost completely dissolved, and extraction of the alkaline solution with ether led to the recovery of only a trace of material. After acidification and ethereal extraction, removal of solvent and drying, a yellow oil was obtained, the methoxyl content of which had fallen from 24·5 % to 14·7 %.

The monomethyl derivative of carolic acid did not go completely into solution. The oil recovered by extraction of the alkaline solution still contained 14·6 % OMe (originally 15·9 %), but the oil recovered after acidification and constituting the larger portion contained only 2·8 % OMe. The portion reacting had therefore been almost completely demethylated. It was found also that shaking the neutral methyl compound with water quickly rendered it acidic, some demethylation occurring.

The dimethyl derivative of carolinic acid almost completely dissolved and the oil recovered from the Na salts after acidification contained 10·0 % OMe (originally 28·4 %). After a further period of hydrolysis the methoxyl content fell to 8·1 %. One methyl group appears therefore to be lost readily and the second group much more slowly.

The monomethyl derivative of α-acetyltetronic acid, which, like the methyl derivatives of carolic and carolinic acids, contains two CO groups in β-positions to the methoxyl group, was hydrolysed with great ease and the product recovered from the Na salts after acidification contained only 3·6 % OMe.

It appears therefore that the methyl derivative of γ-methyltetronic acid, which contains only one CO group in the β-position to the methyl group, behaves much more like a true methyl ether in that it is not readily attacked by NaOH, whereas the methyl groups of the derivatives of α-acetyltetronic and carolic acids and one of the methyl groups of carolinic acid all react like readily hydrolysable methyl ester groupings. The methyl group of α-acetyltetronic acid is however a true methyl ether grouping, is in the β-position of the tetronic acid ring and has two CO groups in β-positions to it, this juxtaposition of potentially acidic groups accounting for the ease with which it is hydrolysed. It seems likely therefore that the readily hydrolysable methyl groups of carolic and carolinic acids are true methyl ether groupings and have the same relative positions. The second methyl group of carolinic acid, which hydrolyses more slowly, must therefore be the true methyl ester grouping.

SUMMARY.

Penicillium Charlesii G. Smith produces from glucose carolic acid ($C_9H_{10}O_4$) and carolinic acid ($C_9H_{10}O_6$). The molecular constitutions of these two acids have been investigated and it has been shown that they are closely related derivatives of γ-methyltetronic acid.

Carolic acid hydrate has been shown to be α-[γ-hydroxybutyryl]-γ-methyltetronic acid and carolinic acid to be α-[β-carboxypropionyl]-γ-methyltetronic acid. Both acids are therefore closely related structurally to ascorbic acid (vitamin C).

A number of derivatives and breakdown products are described.

We gratefully acknowledge much assistance in the preparation of large quantities of carolic and carolinic acids given by Messrs G. Smith and W. K. Anslow.

REFERENCES.

Benary (1909). *Ber. deutsch. chem. Ges.* **42**, 3912.
—— (1910). *Ber. deutsch. chem. Ges.* **43**, 1065.
—— (1911). *Ber. deutsch. chem. Ges.* **44**, 1763.
Birkinshaw and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 139.
Brady (1931). *J. Chem. Soc.* 756.
Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.
Drake and Bronitsky (1930). *J. Amer. Chem. Soc.* **52**, 3715.
Fittig and Chanlaroff (1884). *Liebig's Ann.* **226**, 331.
—— and Ström (1892). *Liebig's Ann.* **267**, 192.
Helferich and Schäfer (1924). *Ber. deutsch. chem. Ges.* **57**, 1911.
Ishikawa and Kojima (1934). *Sci. Rep. Tokyo Bunrika Daigaku* Sect. A, **1**, 43–50. (*Chem. Zentr.* 1934, **1**, 2411).
Kling (1905, 1). *Compt. Rend. Acad. Sci.* **140**, 1457.
—— (1905, 2). *Ann. Chim.* [8], **5**, 554, 556.
—— (1906). *Bull. Soc. Chim.* [3], **35**, 215.
Wedel (1883). *Liebig's Ann.* **219**, 104.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 113.
Wislicenus (1887). *Ber. deutsch. chem. Ges.* **20**, 401.
Wolff (1896). *Liebig's Ann.* **291**, 238.

XXXIX. EGG-WHITE AS SOLE SOURCE OF PROTEIN AND VITAMIN B_2 FOR YOUNG RATS.

By FREDERIK JAN GORTER.

Laan van Altena 47, Delft, Holland.

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When we consider the literature concerning egg-white in nutrition we are struck by the fact that some authors have found toxic properties in dried or fresh egg-white [*e.g.* Boas-Fixsen, 1931; Parsons and Kelly, 1933] and that others have used cooked egg-white as source of protein and vitamin B_2 [*e.g.* Chick, Copping and Roscoe, 1930; György, 1934]. The toxic effect, according to Boas [1927] may be counteracted by a "factor X", occurring in many foodstuffs including dried yeast, and according to Parsons [1930], by liver and yeast. Moreover the damaging property of the egg-white is counteracted by certain treatments, as, for example, thorough coagulation or denaturation by heat [Parsons and Kelly, 1933]. A very slowly acting poison which is counteracted by a normally non-stimulating foodstuff (liver, yeast) may not be considered toxic in the strict sense of the word. Many foodstuffs may become toxic, if fed in too great quantities and if certain counteracting foodstuffs are omitted or reduced. On the other hand, specific deficiency diseases are accompanied, if not caused, by an intoxication with metabolic products. The question whether egg-white, in the dried, fresh, raw or cooked state, is deleterious in the presence of all known food-substances and vitamins, and to what extent the damage may be counteracted by other foodstuffs, containing perhaps a hitherto unrecognised dietary principle, appeared to deserve further investigation. With this aim I undertook the experiments, the results of which are summarised briefly in this paper.

EXPERIMENTAL.

Section I.

Experiments were first made with egg-white subjected to various preparatory treatments, analogous to those already employed by other workers. Albino rats were used and kept in iron cages with mesh floors. The breeding diet contained raw meat, raw vegetables, wheaten bread, wheat, raw milk and a commercial chicken-food, composed of cereal, milk, peanut, meat and fish products and calcium phosphate. This food was administered until the litters were 3 weeks old. The young rats then received, with their mother's milk, an artificial diet containing: 20 % previously coagulated egg-white; 60 % potato-starch; 16 % hardened arachis fat; 4 % salt-mixture [see Stammers, 1909] with traces of $CuSO_4, 5H_2O$. The ration was thoroughly mixed and cooked with 4 parts of tap-water and administered in paste form, supplemented by 2 % cod-liver oil ("jeco-vitol"), to provide vitamins A and D, and by 0·5 % activated acid clay, made from rice-bran according to the method of Jansen and Donath [1927], as source of vitamin B_1. The young rats were weaned when 4 weeks old and the above diet was continued for a further week. When 5 weeks of age, the litters were distributed evenly over three groups, in which the 20 % protein was varied thus: A, 20 % egg-white; B, 20 % purified caseinogen; C, 10 % egg-white + 10 % caseinogen.

In the A diets the treatment of the egg-white was varied as follows:

Diet A1: 20 % raw, dried, powdered Chinese egg-white mixed in the raw state through the cooled food paste.

Diet A2: 20 % (by dry weight) raw, fresh, undried egg-white mixed in the ration.

Diet A3: as A1, but the Chinese egg-white cooked 5 minutes with the ration.

Diet A4: as A2, but the fresh egg-white cooked 5 minutes with the ration.

Diet A5: as A1, but the Chinese egg-white dissolved in water (1½ hours at 30°), then thoroughly coagulated by heating the solution for 3 hours at 80°.

Diet A6: as A5, but the solution denatured by treatment with HCl at p_H 2·4 for 3 days at 37° [Parsons and Kelly, 1933], the reaction being adjusted to p_H 5 by addition of $NaHCO_3$ before mixing with the ration.

In Diets C1 and C2 the 10 % egg-white was respectively dried Chinese and fresh, treated as in Diets A1 and A2.

Diets D3–D6 and E3–E6, contained 20 % egg-white, treated exactly as in Diets A3–A6, but in the D diets 2 % dried brewer's yeast was added and in the E diets 2 % raw, dried horse-liver.

The results of these trials are summarised in Table I. 23 out of 24 young rats on Diets A1 and A2, containing 20 % untreated raw egg-white, dried or fresh, as sole source of protein and vitamin B_2, survived for 42–96 days; cooking the egg-white for a short time (A3 and A4) did not improve matters, as Parsons had

Table I.

All diets contained 60 % potato-starch.

Protein in diet	20 % egg-white						20 % purified caseinogen	10 % egg-white + 10 % purified caseinogen	
	A1	A2	A3	A4	A5	A6	B	C1	C2
No. of rats	15	9	8	12	8	8	13	3	3
Duration of exp. (days)	96	42	62	22	42	20	53	20	12
Average weekly increase in wt. (g.)	4·5	2·5	8	3·5	8	9	4	15	11
Affections and symptoms	Baldness	Eyes Ears Mouth	Paresis	Eyes Ears	Paresis	None	Baldness (2 rats)	None	Eyes (1 rat)
No. of rats dying	0	1	2	4	4	0	4	0	0

Protein in diet	20 % egg-white + 2 % dried brewer's yeast				20 % egg-white + 2 % dried horse-liver		
	D3	D4	D5	D6	E3	E5	E6
No. of rats	8	54	8	8	4	3	2
Duration of exp. (days)	62	22	42	20	33	13	20
Average weekly increase in wt. (g.)	11·5	10·5	9	7·5	7·5	14	15
Affections and symptoms	Eyes Ears Mouth	Eyes Ears	Baldness (1 rat)	None	Baldness	None	None
No. of rats dying	2	12	0	0	0	1	0

already found. Diet A5, in which the egg-white was heated for 3 hours, showed an unexpectedly high rate of mortality (4 out of 8 rats). This I believe to be due to the fact that the egg-white used was not milled as finely as that of the other rations, at least during the first 3 weeks of the experiment; at the end of those 3 weeks half of the rats died. It would seem that the coagulation of a certain part of the egg-white had not been complete. Diet A6, in which the Chinese egg-white was denaturated with HCl, gave the best results.

Diet B (20 % caseinogen) was unsatisfactory in consequence of the deficiency in vitamin B_2.

Diets C1 and C2 (10 % caseinogen with 10 % Chinese dried, or 10 % fresh, egg-white) gave unexpectedly good results during the short time of the trial (12–20 days).

Diets D1–D5, in which the 20 % egg-white was supplemented with 2 % dried brewer's yeast, were not wholly harmless; diets E1–E5, with 2 % dried liver, were rather better during the relatively short time of observation.

The dried egg-white did not generally prove more injurious than the fresh material, but while the latter caused no paresis or baldness, affections of eyes, ears and mouth were often present and the fur was always dirty.

Refection was never seen in these experiments and coprophagy was hindered by the use of wire cage floors of 1 cm. mesh. But, in order to exclude further the chance of refection, most of the above experiments were repeated, using rice-starch and sucrose instead of potato-starch, which according to Boas [1927] has a counteracting effect on the damage caused by egg-white. These results are collected in Table II.

Diets a, b, c, *etc.* correspond to diets A, B, C, described above, with 60 % rice-starch in place of 60 % potato-starch: diets a′, b′, c′, *etc.* contained 20 % of rice starch and 40 % sucrose.

Diets f contained 3 % (by dry weight) of egg-yolk and diets g 20 % of egg-yolk.

Of the diets marked with Greek letters, α contained 40 % egg-white and 40 % rice-starch; α′ 40 % egg-white, no rice-starch and 40 % sucrose; β 40 % purified caseinogen and 40 % rice-starch; γ 20 % egg-white, 20 % purified caseinogen and 40 % rice-starch. Diet γ′ contained 20 % egg-white, 20 % purified caseinogen, no rice-starch and 40 % sucrose; diet δ′ contained 40 % egg-white, 40 % sucrose and 2 % dried brewer's yeast.

In the different experiments summarised in Table II the results regarding growth are not altogether comparable with one another, for the trials of longer duration naturally show lower average growth-rates, owing to the fact that the rats are becoming more nearly adult (see *e.g.* diets α3 and γ3). From these experiments it may, however, be concluded that egg-white in the raw, dried state, is not so very detrimental as source of protein in a diet if all known vitamins are present, including the factors supposed to occur in yeast, egg-yolk and liver, *e.g.* the "factor Y", of Chick and Copping [1930] or "factor X" of Boas [1927; Fixsen, 1930]. If these factors are omitted, damage occurs in some animals, but not in all, unless the egg-white is treated with HCl according to Parsons and Kelly [1933]. The process of drying in itself did not prove to result in damage to the rats, as was thought by Boas [1927].

When the diets contained 60 % cooked potato-starch (Table I) growth was promoted and symptoms prevented rather more effectively than when rice-starch and sucrose (Table II) were used as source of carbohydrates; compare *e.g.* diets A3, A5 with a3, a5 and diet C1 with c1. When, in addition, yeast or liver was given, there was no difference; compare *e.g.* diets D3, D5, E3, E5 with diets

Table II.

Carbohydrate in all diets given as rice-starch or sucrose.

Protein in diet	20 % egg-white						20 % purified caseinogen	
	a1	a'1	a3	a4	a5	a6	b	b'
No. of rats	16	12	13	8	10	6	47	20
Duration of exp. (days)	50	50	62	31	42	40	70	50
Average weekly increase in wt. (g.)	4	5·5	5·5	2	5	4·5	2·5	1·5
Affections and symptoms	Baldness, paresis	Baldness, shaggy coat	Baldness, paresis (2 rats)	Baldness	Baldness, paresis	None	Baldness	Baldness, shaggy coat
No. of rats dying	9	4	7	0	6	2	12	3

Protein in diet	10 % egg-white + 10 % purified caseinogen		20 % egg-white + 2 % dried brewer's yeast					20 % egg-white + 2 % dried horse-liver		
	c1	c6	d1	d3	d4	d5	d6	e3	e5	e6
No. of rats	12	4	12	8	7	8	8	4	2	2
Duration of exp. (days)	40	40	57	62	35	42	20	33	20	20
Average weekly increase in wt. (g.)	6	10·5	12	12·5	9·5	11	10	8	17	14
Affections and symptoms	Baldness, paresis	Baldness	None	Eyes Ears (2 rats)	Slight baldness	Baldness	None	Baldness (1 rat)	None	None
No. of rats dying	4	1	1	2	0	0	0	0	1	0

Protein in diet	20 % egg-white + 3 % and 20 % egg-yolk		40 % egg-white				40 % purified caseinogen
	f4	g4	α1	α'1	α3	α4	β
No. of rats	8	8	4	3	8	4	10
Duration of exp. (days)	31	31	40	33	76	20	28
Average weekly increase in wt. (g.)	7	15	6	4	(2)	10	8
Affections and symptoms	None	None	Baldness (1 rat)	Shaggy coat	Shaggy coat	Shaggy coat	Shaggy coat
No. of rats dying	0	0	2	0	0	0	0

Protein in diet	20 % egg-white + 20 % purified caseinogen				40 % egg-white + 2 % dried brewer's yeast	
	γ1	γ'1	γ2	γ3	δ'1	δ'4
No. of rats	4	6	6	10	1	8
Duration of exp. (days)	24	28	8	76	19	20
Average weekly increase in wt. (g.)	9	11	12	(5·5)	10	14·5
Affections and symptoms	Baldness	Shaggy coat	None	Shaggy coat	None	Shaggy coat
No. of rats dying	0	0	0	0	0	0

d3, d5, e3, e5. These results confirm the conclusion of Boas [1927] that a "factor X", occurring in potato-starch, as well as in yeast, commercial caseinogen and several other foodstuffs, was able to counteract the toxic properties of egg-white in the diet.

During the above experiments (section I) my attention was attracted to the extraordinarily good growth on diets C, c and γ, which contained both purified caseinogen and egg-white as sources of protein. Boas-Fixsen found that factor X occurred to a relatively small extent in commercial caseinogen, but although a special growth-promoting property has been ascribed to commercial "light white casein" (Coward) no such property has been shown to exist in "purified" caseinogen. Further evidence on this subject is described in the following section.

Section II.

In order to accustom 124 young rats 3 weeks old to egg-white, 5 % (dry weight) of fresh rapidly coagulated egg-white was added to a b-ration, which already contained 20 % purified caseinogen, which had been purified by washing the caseinogen in a mixture of 10 l. "Monster" tap-water, 5 l. 96 % alcohol and 28 ml. *N* HCl (p_H 4·1 measured by glass-electrode) during 1 week, subsequently washing in acid 85–90 % alcohol (p_H 5·1) during 1 week and finally extracting with ether in a Soxhlet-apparatus for 2 weeks. After 2 weeks, when 5 weeks old, the rats were distributed over 2 groups, one of which received 20 % purified caseinogen as sole source of protein (39 rats), the other 20 % (dry weight) of fresh, rapidly coagulated egg-white. For both groups the increase in weight during the following 6 weeks (18–20 g.) was about equal to that of the comparable groups in section I above (a4, b see Fig. 1 and Table II) and in both the greater part of this increase (*ca.* 12 g.) occurred in the first 2 weeks, that is in the 6th and 7th weeks of life (Fig. 1). The young rats described above in section I had never shown this peculiarity; those fed on diet a4 increased by 2 g. during the first 10 days and those on diet b by 3·5 g. during the first 2 weeks, *i.e.* by rather less than the average weekly rate of growth shown over the longer period, which was 2 and 2·5 g. respectively. It was evident that in the present instance the previous presence in the rations of egg-white and purified caseinogen exerted a beneficial effect on the growth even for 2 weeks after one of them had been omitted. If this is true, the growth should also be enhanced for a period of about 2 weeks when a change is made from 20 % egg-white to 20 % purified caseinogen or *vice versa*. Special experiments were made to test this. 47 rats 3 weeks old received diet a4 (egg-white), 65 others diet b (caseinogen). When 4 weeks old they were weaned and, when 5 weeks old, distributed over 4 groups; some which had been fed on diet a4 (egg-white) now received diet b (caseinogen) and some which had been fed on diet b, now received diet a4. The remainder in each

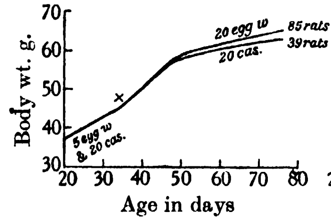

Fig. 1.

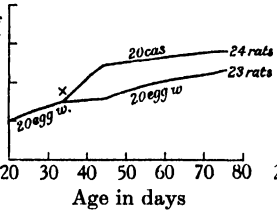

Fig. 2.

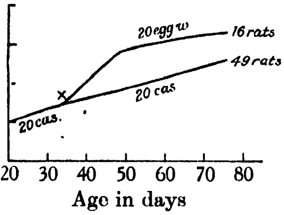

Fig. 3.

× Denotes division of the rats into 2 groups, each group receiving a different kind of protein.

group were continued on the previous diet. The result was a clear-cut corroboration of my expectation (Figs. 2 and 3).

When the diet during the 4th and 5th weeks of life had contained 20 % egg-white, the average growth on the changed diet (caseinogen, 24 rats) was 9·5 g. during the first 10 days, while that on the unchanged diet (23 rats) was 0·5 g. When the diet during the 4th and 5th weeks had contained 20 % purified caseinogen, the average growth on the unchanged diet (49 rats) was 3·5 g. during the 6th and 7th weeks and on the changed diet (egg-white) 12·5 g. (16 rats). Thus, in either case when the protein of the diet was altered, the growth was at first stimulated, presumably because the changed protein provided some factor absent from the previous one. After 10–14 days, the rate diminished (1–3 g. weekly), presumably because the reserve of some factor present in the first protein, but absent from the second, was becoming exhausted. When, on the contrary, the protein was not altered, there was no improvement in either growth or condition. Change in appetite could not have played any part, for the food intake was kept the same in all groups by giving a relatively small supply every day, so that each rat could eat it all.

We may conclude, therefore, that purified caseinogen, preferably in combination with potato-starch, can partially replace the growth-promoting action of yeast, liver or egg-yolk when added to diets containing egg-white as source both of protein and vitamin B_2. Vitamins A, B_1 and D were always given in sufficient doses. In such diets both purified caseinogen and egg-white are required to maintain an appreciable though subnormal growth; the first supplies an unrecognised food principle, the second vitamin B_2.

Section III.

In the following experiments additional evidence is given that growth may be almost completely restored by incorporation of 40 % purified caseinogen (see group 3) into the ration containing egg-white as source of protein, and that the essential dietary factor, which is thus added, is not extractable by washing the caseinogen with dilute acid alcohol (27 %), acid, 85–90 % alcohol or ether.

Two litters of young rats during the 4th and 5th weeks of life received a diet containing as much as 50 % (dry weight) egg-white, this high proportion being at the expense of the potato-starch, which was reduced to 30 %. In other respects the diet resembled diet A3 of section I. No symptoms of "proteintoxicose" [Glanzmann, 1934] caused by the large amount of rapidly coagulated, previously dried egg-white were to be seen during these 2 weeks. (Glanzmann often observed paresis and death within 2 days with dried buttermilk.) The litters were then distributed over three groups, 6 rats in each, maintained on diets containing different proteins, with results as follows:

Group 1: 50 % egg-white; weekly growth 10·5 g. during 3 weeks.

Group 2: 50 % purified caseinogen; weekly growth 12 g. during 3 weeks.

Group 3: 10 % egg-white and 40 % purified caseinogen; weekly growth 19·5 g. during 4 weeks.

In group 3 growth was almost maximum, because the two factors contained in egg-white and in purified caseinogen, respectively, supplemented each other in their growth-promoting actions. Maximum growth (25 g. weekly, as was obtained with the breeding diet) was only secured by the addition of 3 % dried yeast (suggesting the further action of the factor Y of Chick and Copping [1930]).

In groups 1 and 2 the weight increase stopped after 1 month; in group 3 it diminished, but remained almost maximum during 2 months, even when no yeast was added, showing that not only "factor Y" but also another factor may be growth-promoting when added to the egg-white rations. The rats of group 1 (50 % egg-white) when 6 weeks old, developed bald patches on head, neck and back, giving them a rather moth-eaten appearance. The rats of group 2 (50 % purified caseinogen), after recovering from a slight degree of baldness evidently due to the preceding feeding with egg-white during the 4th and 5th weeks, developed rather shaggy coats when 8 weeks old. The rats of group 3 remained in good condition, but after 2 months incipient depigmentation sometimes appeared when pied rats were used [Gorter, 1934]. One may conclude that the baldness is associated with egg-white feeding and that this can probably be prevented by a dietary factor which occurs to a relatively small extent in purified caseinogen.

DISCUSSION.

The question arises, whether 20 % egg-white is to be regarded as an incomplete, or as a toxic, source of protein and whether it contains enough vitamin B_2 for growth. Mitchell [1925] found 18 % coagulated egg-white to be a satisfactory source of protein; in this work dried whole yeast was the source of B-vitamins, but this contains protein also. Boas [1927; Boas-Fixsen, 1931] and Parsons [1930] described, however, specific ill-effects caused by 20–66 % egg-white (dry weight) in the diet; as source of B-vitamins they used marmite (Boas) and wheat embryo or an extract of wheat embryo.

According to Parsons and Kelly [1933] the toxic effect of egg-white cannot be due to an amino-acid deficiency; for when thoroughly denatured (with HCl), 20–66 % egg-white in a diet always proved harmless. The possible influence of the protein provided by the 10 % whole wheat embryo used in their most successful growth experiments should, however, be remembered, as a hint that an amino-acid deficiency of the egg-white is still possible.

In respect of the vitamin B_2 quality of egg-white, Chick, Copping and Roscoe [1930] concluded that 20 % egg-white (dry weight), as sole source of protein and of vitamin B_2, provided an ample supply of vitamin for a few weeks only.

If egg-white be regarded as adequate for provision of vitamin B_2 there must be some other nutritive fault of diets containing 20 % coagulated egg-white as sole source of protein and vitamin B_2, a defect which, however, does not influence the growth for the first 2 weeks. If purified caseinogen is also present in sufficient quantity in the diet (preferably in combination with potato-starch), the damage associated with egg-white does not appear for a period of 3 months. The hypothetical dietary factor, occurring in purified caseinogen, possibly an amino-acid, is distinguished from all known B-vitamins except B_3 [see O'Brien, 1934] by its relative insolubility in acid dilute alcohol. It may be connected with the equally insoluble factor X [Boas, 1927; Boas-Fixsen, 1931; Glanzmann, 1934; Lease and Parsons, 1934, 1] which counteracts the deleterious effect of egg-white in the dried raw state, or perhaps with the dietary factor described by Coward *et al.* [1929] in "light white casein" (see also Mapson [1933]). The latter, however, was not present in (purified) "Glaxo casein" and was soluble in 96 % alcohol.

Lease and Parsons [1934, 2] recently extracted a factor curative of the egg-white dermatitis in rats from kidney or liver, after previous digestion with papain. I have not yet tried this method with caseinogen.

SUMMARY.

1. The toxic effects of egg-white, when present as sole source of protein and vitamin B_2 in a diet, which cause the well-known skin disorder in young rats, can be counteracted by substitution of purified caseinogen for part of the egg-white.

2. The dietary factor contained in caseinogen and lacking in egg-white is present also in liver, yeast and egg-yolk; it is insoluble in dilute or strong acid alcohol or in ether.

3. While differing in solubility from the known B-vitamins and from the dietary factor in commercial caseinogen described by Coward *et al.*, it would appear to be similar in distribution and function to the "factor X" described by Boas, and partly also to the "factor Y" described by Chick *et al.* The possibility that it may be an amino-acid is not excluded.

REFERENCES.

Boas (1927). *Biochem. J.* **21,** 712.
Boas-Fixsen (1931). *Biochem. J.* **25,** 596.
Chick and Copping (1930). *Biochem. J.* **24,** 1764.
—— Copping and Roscoe (1930). *Biochem. J.* **24,** 1748.
Coward, Key and Morgan (1929). *Biochem. J.* **23,** 695.
Fixsen (1930). *Biochem. J.* **24,** 1794.
Glanzmann (1934). *Z. Vitaminforsch.* **3,** 2.
Gorter (1934). *Nature,* **134,** 382.
György (1934). *Nature,* **133,** 498.
Jansen and Donath (1927). *Mededeel. Dienst Volksgez. Ned. Ind.* **1,** 190.
Lease and Parsons (1934, 1). *Biochem. J.* **28,** 2109.
—— —— (1934, 2). *J. Biol. Chem.* **105,** li.
Mapson (1933). *Biochem. J.* **27,** 1061.
Mitchell (1925). *Amer. J. Physiol.* **64,** 359.
O'Brien (1934). *Biochem. J.* **28,** 926.
Parsons (1930). *J. Biol. Chem.* **90,** 351.
—— and Kelly (1933). *J. Biol. Chem.* **100,** 645.
Stammers (1909). *Biochem. J.* **15,** 489.

XL. THE IRON AND COPPER CONTENTS AND THE HAEMOPOIETIC ACTIVITIES OF STOMACH AND LIVER PREPARATIONS.

By HAROLD JACKSON, LOUIS KLEIN
and JOHN FREDERICK WILKINSON.

From the Department of Clinical Investigations and Research, University and Royal Infirmary, Manchester.

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The modern treatment of pernicious anaemia with preparations of liver or hog's stomach has led to much speculation as to the precise cause of the success of the treatment, and many laborious fractionation experiments have been carried out with the object of isolating and studying the properties of the active anti-anaemic substances presumed to be present in these organs. These active principles have not yet been isolated in pure form; only concentrated preparations or fractions are known whose haemopoietic activity can be determined solely by the clinical method of observing their effects in cases of pernicious anaemia.

The investigations of Cohn *et al.* [1928; 1930] suggest that the active principle in liver is a nitrogenous base, possibly an ω-amino- or imino-acid. Further, the work of two of us [Wilkinson and Klein, 1932; 1933; 1934; Klein and Wilkinson, 1933; 1934] has shown that the anti-anaemic factor in hog's stomach, haemopoietin, is not identical with the liver principle but is probably an enzyme, which, reacting with a substrate present in the normal diet, produces a substance which from its chemical and therapeutic properties appears to be identical with the so-called active liver principle; this liver principle is utilised for the formation of red blood cells by some mechanism at present unknown.

The liver therapy had its origin in the classical experiments of Whipple *et al.* [1920], who, selecting liver owing to its relatively high iron content, showed that it was the most potent foodstuff in causing blood regeneration in dogs which had been kept at a constant low haemoglobin level by bleeding. This work on post-haemorrhagic anaemia induced Minot and Murphy [1926] to try the effect of liver feeding on cases of pernicious anaemia. The success they had with this diet was later paralleled by the even more striking results that followed the treatment of pernicious anaemia with preparations of hog's stomach [Wilkinson, 1930; 1931].

The preparations of liver and stomach used in the treatment of pernicious anaemia contain small amounts of iron and copper and it has been suggested in many quarters [*cf.* for example Schultze, 1932] that they owe their value to the presence of these mineral constituents. Iron, of course, is known to be essential for haemoglobin formation and has been used with great success in the treatment of the hypochromic microcytic anaemias.

The possibility that copper may also be an active blood-regenerating agent or may stimulate haemoglobin formation has been suggested by many workers [*cf.* McHargue *et al.*, 1928; Flinn and Inouye, 1929; Hart *et al.*, 1928; Waddell *et al.*, 1929, 1, 2; Elvehjem, 1932]. According to Warburg and Krebs [1927], Schönheimer and Oshima [1929], Guillemet [1931], McFarlane [1932], Locke *et al.* [1932] and Tompsett [1934, 1], human blood contains small amounts of copper, the amount being of the order of 0·1–0·2 mg. Cu per 100 ml. McHargue [1925] suggests that it may also perform important functions in the absorption and transfer of oxygen in the respiration of mammals. Indeed, copper is an essential constituent of haemocyanin, the respiratory pigment of certain molluscs and crustacea. Beard and Myers [1928], working on nutritional anaemia of rats, found that traces of copper added to iron brought about regeneration of haemoglobin in less time than the same dose of iron alone. Mills [1930], among others, reports the successful use of copper and iron in idiopathic anaemia of adults.

The experimental evidence so far accumulated appears to suggest that iron and copper may play an important part in certain forms of anaemia, though whether they do so in pernicious anaemia is open to question. The object of the present work was to determine whether the quantities of iron and copper present in various liver and stomach preparations and fractions used in the treatment of pernicious anaemia could be correlated with the haemopoietic activities of the fractions as judged by clinical trial on cases of this disease.

EXPERIMENTAL.

In view of the relatively small quantities of iron and copper present in the preparations used, it was decided to employ colorimetric methods of estimation using a Klett colorimeter, and throughout the work, distilled water and analytical reagents shown to be free from iron and copper were used. In view of the fact that there appears to be some vagueness in the literature as to the exact procedure in these methods, it has been thought advisable to describe our experimental technique at some length.

Ashing of preparations. The following dry ashing method was found to be the most satisfactory. Porcelain crucibles (2 in. in diameter) of the best Berlin make were soaked in alcoholic sodium acetate and then evaporated to dryness several times with concentrated hydrochloric acid in order to remove any iron and copper from the glaze [*cf.* Elvehjem and Lindow, 1929]. In the case of stomach preparations, 5 g. samples were generally used, but, owing to scarcity of material, 1 g. or even less of some of the liver fractions was used. The crucible was heated over a very low flame until visible charring commenced, when concentrated sulphuric acid (about 10–15 drops per 5 g. sample) was carefully added. Heat was then gently applied until a carbonaceous mass was obtained. A further similar quantity of acid was then added and heating continued for approximately an hour, when ashing was usually complete. If this were not so, the crucible was allowed partially to cool, one drop of acid added to any portion where carbonaceous material remained and the crucible reheated for a short period.

Extraction of ash. This was carried out by heating for a short time almost to boiling point with concentrated hydrochloric acid (5–10 ml.) over a microburner. The acid solution was then measured into a volumetric flask (50 ml. for a 5 g. sample, 20 ml. for smaller samples) and further extractions were made with similar quantities of water. The solution was made up to volume after cooling, filtered through a sintered glass filter and was then ready for the iron and copper estimations.

Preparation of standard iron and copper solutions. Iron standards were prepared as follows:

(*a*) Ferric alum A.R. (0·8630 g.) was dissolved in 50 ml. water, 20 ml. of 10 % hydrochloric acid were added to prevent hydrolysis and the solution was then diluted to 1 litre (1 ml. = 0·1 mg. iron). The purity of the salt was confirmed by gravimetric analysis.

(*b*) Pure iron wire (0·0639 g.) was dissolved in 50 ml. of 10 % hydrochloric acid, oxidised with 5 ml. hydrogen peroxide (20 vols.) and the solution boiled and made up to 500 ml. This solution should contain 0·128 mg. iron per ml., and colorimetric estimation by the thiocyanate method (following the details given below), taking the ferric alum solution as standard, gave a value of 1 ml. = 0·129 mg. iron.

The standard copper solution contained 0·3928 g. copper sulphate A.R. ($CuSO_4$, $5H_2O$) per litre (1 ml. = 0·1 mg. copper).

Determination of iron by the thiocyanate method. The intense wine-red coloration produced by the action of thiocyanates on ferric salts (sensitivity 1 in 1,000,000) has been the basis of the common methods in use for the determination of iron in biological material. Many workers recommend extraction of the colouring matter with amyl alcohol before colorimetric comparison [*cf.* Kennedy, 1927; Elvehjem, Kemmerer *et al.*, 1929; McFarlane, 1932], whilst others consider this procedure to be unnecessary [*cf.* Brown, 1922; Wong, 1923; Rees, 1933]. We find, however, that very poor colour matches are obtained if readings are made directly without extraction. On the other hand, with the extraction method, it is sometimes necessary to dry the amyl alcoholic extracts with sodium sulphate owing to their turbidity.

An accurately measured aliquot portion (usually 5 or 10 ml.) of the unknown solution was measured into a 100 ml. cylindrical separating funnel, the iron oxidised to the ferric condition by addition of 2 drops of concentrated nitric acid and water added from a burette to a volume of 20 ml. This was followed by an exact volume of amyl alcohol (usually 20 ml. but varying according to the colour developed) and 5 ml. of 20 % potassium thiocyanate solution. Extraction was then carried out immediately, the amyl alcoholic extract dried if necessary, by filtration through a thin layer of anhydrous sodium sulphate (about 1 g.) on a sintered glass filter, and the depth of colour compared with that of a standard iron solution freshly made up in an identical manner.

The minimum concentration of iron which gave a sufficiently intense colour for convenient reading (standard at 20 mm. depth) on the colorimeter was $2{\cdot}5 \times 10^{-6}$ mg. iron per ml. amyl alcohol solution (*i.e.* ≡ 0·5 ml. of standard extracted with 20 ml. amyl alcohol).

Determination of iron by the thiolacetic acid method. Lyons [1927] has shown that ferric or ferrous salts give a purple coloration with thiolacetic acid (sensitivity 1 in 5,000,000) which can be utilised for the quantitative estimation of iron, and Hanzal [1933], Burmester [1934] and Tompsett [1934, 2] have applied the test to the determination of iron in biological material. The main objections to the use of this reaction as a basis for a colorimetric method of analysis are (*a*) the instability of the coloration which fades in the course of a few hours, owing apparently to the presence of copper, and (*b*) the formation of a fine precipitate of alkaline earth hydroxides and phosphates in the ammoniacal solution which makes readings difficult. We find, however, that when the solutions are allowed to stand for 15 minutes and then filtered through a sintered glass filter, the results are in good agreement with those obtained by the thiocyanate method.

An aliquot portion of the unknown solution was diluted with water to 20 ml., 1 drop of thiolacetic acid added, followed by 1 ml. of ammonia (sp. gr. 0·88). It was occasionally necessary to add 2–3 ml. of ammonia when the aliquot portion of the unknown solution was large. The depth of colour was then compared with that of 1 ml. of the standard iron solution treated similarly.

The minimum concentration of iron which gave a sufficiently intense colour for convenient reading (standard at 30 mm.) on the colorimeter was $4{\cdot}0 \times 10^{-6}$ mg. iron per ml. solution (*i.e.* ≡ 1 ml. of standard solution made up to a total volume of 25 ml.).

Estimation of copper in the presence of iron. The most delicate colour test for copper is the reaction with sodium diethyldithioçarbamate [Callan and Henderson, 1929], which gives a golden-yellow coloration with dilute solutions of copper salts (sensitivity 1 in 100,000,000). Since iron also gives a coloration with this reagent, a modification of the original method first suggested by Haddock and Evers [1932] has been used. It was found, however, that carbon tetrachloride as used by the latter authors was unsatisfactory as an extractant since several extractions were necessary for the complete removal of the colour developed and, moreover, the solutions in this solvent were not very stable. The use of amyl alcohol, introduced by McFarlane [1932] in place of carbon tetrachloride, had none of these objections and has recently been recommended by Tompsett [1934, 1].

The unknown solution (25 ml. or an aliquot portion made up to 25 ml. with water) was introduced into a 100 ml. cylindrical separating funnel and 2 drops of concentrated nitric acid were added. This was followed by 1 g. of citric acid and, after solution had taken place, 4 ml. of ammonia (sp. gr. 0·88) were added, giving a p_H greater than 9·0. The total volume was then made up to 40 ml. with water from a burette. A known volume of amyl alcohol (usually 20 ml.) followed by 5 ml. of a 0·1 % solution of sodium diethyldithiocarbamate was now added and the whole vigorously shaken for a short time. After separation had taken place, the aqueous layer was removed. It was very occasionally found necessary to dry the amyl alcohol layer as in the iron estimation described above. Comparison was then made with a standard copper solution (0·5 ml. or 1 ml.) treated in the same way as the unknown.

In many cases where the quantity of available material was small (0·35–0·5 g.), the hydrochloric acid solution of the ash was made up to 25 ml., and 20 ml. were subsequently extracted with 10 ml. of amyl alcohol.

The minimum concentration of copper giving a sufficiently intense colour for convenient reading (standard at 30 mm.) was $2{\cdot}5 \times 10^{-6}$ mg. copper per ml. of amyl alcohol solution (*i.e.* ≡0·5 ml. of standard extracted with 20 ml. amyl alcohol).

DISCUSSION OF RESULTS.

The results of our determinations of iron and copper in various products derived from hog's stomach and from various livers are shown in Tables I–III. Estimations were carried out in duplicate except in the cases of one or two human liver fractions where the amount available did not permit of duplicate ashing.

All the preparations have been tested for haemopoietic activity on specially controlled cases of pernicious anaemia, the stomach and ox liver preparations being administered orally and the human liver fractions by intramuscular injection. Haemopoietically active products produced a "peak" in the number of reticulocytes in the circulating blood in the course of a few days, followed by a fall to normal values, and this was accompanied by an increase in the number of red blood cells and in the percentage of haemoglobin [*cf.* Wilkinson, 1932; 1933]. The clinical material used for this purpose conformed to certain rigid criteria already laid down by one of us, and the clinical details have already been published elsewhere [Wilkinson, 1932; Klein and Wilkinson, 1933; 1934; Wilkinson and Klein, 1934].

In considering the determinations on the desiccated stomach preparations (Table I) it will be noted that the copper content was very small compared with the amount of iron present and showed quite a reasonable constancy, varying only between 1·3 and 2·0 mg. per 100 g. The iron content of these products was very much higher and, moreover, showed variations between wider limits. One of the preparations (A) gave a much higher figure for iron than the others. The data presented appear to show conclusively that there is but little difference in the metallic contents of the haemopoietically active and inactive preparations, the inactive preparation F, for example, containing practically the same amount of iron and copper as the active preparation H. The haemopoietic inactivity of certain desiccated stomach preparations is due then not to any deficiency or excess of iron or copper but to the methods of treatment during their manufacture, which causes inactivation of the sensitive gastric enzyme, haemopoietin [Wilkinson, 1932]; this is further manifest from the fact that gentle heating destroys haemopoietin but has no influence on the iron and copper contents of the preparations.

Table I.

Preparation	Haemopoietic activity	Mean values mg. per 100 g. preparation: Iron	Mean values mg. per 100 g. preparation: Copper
	Commercial desiccated hog's stomach		
A	Active	33·2	1·7
B	Inactive	17·5	1·7
C	Active	10·5	1·8
D	Active	12·4	1·9
E	Active	16·4	1·3
F	Inactive	11·5	1·2
G	Inactive	8·5	2·0
H	Active	11·5	1·5
	Fractions from hog's stomach press juice		
Fraction P5	Active	95·8	3·3
Fraction P5 (i)	Inactive	97·3	5·9
Fraction P5 (ii)	Active	33·2	7·0

Table I also shows the iron and copper contents of three stomach fractions prepared by two of us from the expressed juice of hog's stomach [Wilkinson and Klein, 1932; Klein and Wilkinson, 1933].

Fraction P5 was obtained by precipitating the press juice directly with alcohol. Fraction P5 (i) represented the precipitate rich in pepsin obtained by dissolving fraction P5 in $N/10$ hydrochloric acid and bringing to p_H 4·2 by addition of alkali. Fraction P5 (ii) was the pepsin-free precipitate obtained by adding alcohol to the filtrate from the preparation of fraction P5 (i).

Two of these fractions (P5 and P5 (i)) had very high iron contents, though one was active and the other was not. Moreover, the active fraction P5 (ii) had only one-third of the amount of iron present in the inactive fraction P5 (i), which provides further evidence of lack of any relationship between haemopoietic activity and metallic content. In point of fact, most of the iron present in these fractions was probably derived from the metal press used in the preparation of the press juice.

Table II gives the results obtained with an ox liver extract and an extract prepared by digesting ox liver with hog's stomach—both preparations were for oral use and were clinically very active, the latter preparation being more active than the former. In one of these fractions, the quantity of copper was in

Table II. *Preparations from ox liver and hog's stomach.*

Preparation	Haemopoietic activity	Mean values mg. per 100 g. preparation: Iron	Mean values mg. per 100 g. preparation: Copper
Liver extract S.P.I. (7·8 g. ≡ ½ lb. liver)	Active	23·6	43·9
Liver—hog's stomach L.S.I. (6·4 g. ≡ ½ lb. liver)	Active	12·3	9·6

excess of the iron content whereas in the other the converse held. Here again there appears to be no evidence of any parallelism between the haemopoietic activities and the contents of iron and copper.

The results obtained with liver fractions prepared for intramuscular injection from various normal and abnormal human livers as described elsewhere [Wilkinson and Klein, 1934] are shown in Table III.

Table III. *Fractions from human livers.*

Clinical source of liver	Fraction	Haemopoietic activity	Mean values mg. per 100 g. fraction: Iron	Mean values mg. per 100 g. fraction: Copper
Untreated pernicious anaemia	L.I. 3	Inactive	<1·0	8·6
	L.I. 19	Inactive	4·3	9·1
Partially treated pernicious anaemia	L.I. 1	Slight	<1·0	15·7
Remitting pernicious anaemia	L.I. 4	Active	—	4·3
	L.I. 13	Active	4·9	15·7
Normal	L.I. 7	Active	4·0	2·9
	L.I. 12	Active	18·9	5·1
Haemolytic anaemia	L.I. 22	Active	5·8	11·3
Polycythaemia rubra	L.I. 14	Very active	<1·0	7·9

It will be seen that there is a very wide variation in the copper and iron contents of the different liver fractions and that no correlation exists between their clinical potencies and contents of these metals.

Thus, in the haemopoietically active fractions iron varied from <1·0 to 18·9 mg. while the copper was between 2·9 and 15·7 mg.; the inactive or slightly active ones had ranges of <1·0 to 4·3 mg. for iron and 8·6 to 15·7 mg. for copper.

It is clear, therefore, that the haemopoietic activities of these liver fractions are quite independent of the iron and copper they contain, but it should be appreciated that these values do not represent the metallic contents of the original fresh tissues, which values do not concern the main objects of this paper.

In passing it may be noted, however, that Meyer and Eggert [1932] found that only part of the iron and copper in livers from various mammals could be extracted with water. These metals tended to accumulate in the fraction which was precipitated by 67 % alcohol, a fraction which Whipple *et al.* [1930] found effective in microcytic hypochromic anaemias. On the other hand, the fraction subsequently obtained by precipitation in 95 % alcoholic solution (corresponding with the fraction G shown by Cohn *et al.* [1928] to be effective in pernicious anaemia) contained less copper and very little iron. These authors concluded that the substance in liver effective in pernicious anaemia has no relation to the iron and copper content of the liver. Moreover, Cohn *et al.* [1930] state that they have succeeded in preparing from liver a fraction containing no iron which is very effective in the treatment of pernicious anaemia.

The evidence, then, provided by our own data and those of these other workers goes to show that the iron and copper contents of the preparations of liver and stomach used in the treatment of pernicious anaemia bear no relationship to their haemopoietic potencies, and there is no support for the view that the anti-(pernicious) anaemic factors owe their activities to the presence or absence of these metals. Further, the total amounts of iron and copper in these preparations are infinitesimal in comparison with the quantities ingested in the ordinary diet and, indeed, in comparison with the normal therapeutic dose used in the treatment of other anaemias.

SUMMARY.

1. Determinations have been made of the quantities of iron and copper in various clinically tested preparations of stomach and liver used in the treatment of pernicious anaemia.

2. Iron was determined colorimetrically by slight modifications of existing methods involving the use of potassium thiocyanate and thiolacetic acid respectively.

3. Copper was estimated colorimetrically by a modification of the sodium diethyldithiocarbamate method.

4. The results do not indicate any obvious relationship between the iron and copper contents and the haemopoietic potencies as judged by clinical trial on cases of pernicious anaemia.

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REFERENCES.

Beard and Myers (1928). *Proc. Soc. Exp. Biol. Med.* **26**, 510.
Brown (1922). *J. Amer. Chem. Soc.* **40**, 423.
Burmester (1934). *J. Biol. Chem.* **105**, 189.
Callan and Henderson (1929). *Analyst*, **54**, 650.
Cohn, McMeekin and Minot (1930). *Trans. Assoc. Amer. Physicians*, **45**, 343.
—— Minot, Alles and Salter (1928). *J. Biol. Chem.* **77**, 325.
Elvehjem (1932). *J. Amer. Med. Assoc.* **98**, 1047.
—— Kemmerer, Hart and Halpin (1929). *J. Biol. Chem.* **85**, 92.
—— and Lindow (1929). *J. Biol. Chem.* **81**, 435.
Flinn and Inouye (1929). *J. Biol. Chem.* **84**, 101.
Guillemet (1931). *Compt. Rend. Soc. Biol.* **108**, 32.
Haddock and Evers (1932). *Analyst*, **57**, 495.
Hanzal (1933). *Proc. Soc. Exp. Biol. Med.* **30**, 846.
Hart, Steenbock, Waddell and Elvehjem (1928). *J. Biol. Chem.* **77**, 797.
Kennedy (1927). *J. Biol. Chem.* **74**, 385.
Klein and Wilkinson (1933). *Biochem. J.* **27**, 600.
—— —— (1934). *Biochem. J.* **28**, 1684.
Locke, Main and Rosbash (1932). *J. Clin. Invest.* **11**, 527.
Lyons (1927). *J. Amer. Chem. Soc.* **49**, 1916.
McFarlane (1932). *Biochem. J.* **26**, 1022.
McHargue (1925). *Amer. J. Physiol.* **72**, 583.
—— Healy and Hill (1928). *J. Biol. Chem.* **78**, 637.
Meyer and Eggert (1932). *J. Biol. Chem.* **99**, 265.

Mills (1930). *Can. Med. Assoc. J.* **22**, 175.
Minot and Murphy (1926). *J. Amer. Med. Assoc.* **87**, 470.
Rees (1933). *Analyst*, **58**, 384.
Schönheimer and Oshima (1929). *Z. physiol. Chem.* **180**, 249.
Schultze (1932). *Klin. Woch.* **11**, 597.
Tompsett (1934, 1). *Biochem. J.* **28**, 1544.
—— (1934, 2). *Biochem. J.* **28**, 1536.
Waddell, Steenbock and Hart (1929, 1). *J. Biol. Chem.* **83**, 243.
—— —— —— (1929, 2). *J. Biol. Chem.* **84**, 115.
Warburg and Krebs (1927). *Biochem. Z.* **190**, 143.
Whipple, Robscheit and Hooper (1920). *Amer. J. Physiol.* **53**, 236.
—— Robscheit-Robbins and Walden (1930). *Amer. J. Med. Sci.* **179**, 628.
Wilkinson (1930). *Brit. Med. J.* i, 236.
—— (1931). *Brit. Med. J.* i, 85.
—— (1932). *Brit. Med. J.* i, 325.
—— (1933). *Proc. Roy. Soc. Med.* **26**, 1341.
—— and Klein (1932). *Lancet*, i, 719.
—— —— (1933). *Lancet*, ii, 629.
—— —— (1934). *Quart. J. Med., N.S.* **3**, 341.
Wong (1923). *J. Biol. Chem.* **55**, 421.

XLI. THE FORMATION OF CARBOHYDRATE FROM FAT IN THE LIVER OF THE RAT.

BY CHALMERS LAUGHLIN GEMMILL[1] AND
ERIC GORDON HOLMES.

From the Biochemical Laboratory, Cambridge.

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THERE have been many attempts to test whether or not fat can be transformed into carbohydrate in the animal body. The majority of the experiments have been negative, and the few which have given positive results have met with criticism. Burn and Marks [1926] reported an increase in glycogen in a perfused liver taken from a cat or dog after a high fat diet. At the same time they observed an increase in the sugar of the perfusion fluid. Gregg [1933] repeated these experiments and found, in the majority of his experiments, a decrease of liver glycogen. He explained the results of Burn and Marks and similar results obtained by earlier investigators on the grounds that feeding fat to dogs and cats does not completely rid the liver of glycogen. Also, he found that the distribution of the glycogen in the liver was so variable that a sample from one lobe did not give any information concerning the carbohydrate content of the whole liver.

Apart from perfusion experiments, evidence of this transformation is provided by the increase of glycogen found to occur in the livers of intact animals fed on fat diets. Takao [1926], Burn and Ling [1929] and Magnüsson [1929] have reported high glycogen values under this condition. For example, Burn and Ling found values as high as 5·0 % after 96 hours of fat feeding. In contrast to these results are those of Bodey *et al.* [1927], Gregg [1931] and Greisheimer [1931]. These observers failed to find such high percentages of glycogen as the workers quoted above, although in the majority of their experiments they did find a small but definite increase. Gregg [1931] claimed that this rise was due to the glycerol content of the fat. In support of this view, he found that when a rat was given the soaps of butter fat, there was no increase in the glycogen content over that shown by his series of fasting rats. As Gregg reported only one experiment of this nature, his conclusion is only suggestive.

Another type of evidence has been obtained from a study of the D/N ratio in depancreatised dogs. Soskin [1929] fed fat and lecithin to diabetic dogs. He found in 3 out of 15 experiments an increase in sugar in the urine. He concluded from this evidence that gluconeogenesis from fatty acids was proven. Page and Young [1932] were unable to obtain any increase in urinary sugar following an intravenous injection of lecithin into phlorhidzinised dogs. These experiments do not contradict those of Soskin, as it is now well recognised that the metabolism of a diabetic animal and a phlorhidzinised one are not the same; it is very difficult to transfer the results from one type of experiment to the other. The three positive experiments of Soskin are the most direct evidence that we have had, up to the present time, for transformation of fat into carbohydrate in the animal body.

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[1] Associate in Physiology, Johns Hopkins University, School of Medicine.

The respiratory quotient of man and animals on fat diets has given suggestive evidence for this transformation. This subject has been studied recently by Hawley *et al.* [1933]. They found many respiratory quotients of less than 0·70 for men on a diet of cream. They concluded that these low quotients could be explained by the supposition that the process of desaturation of fats occurred more rapidly than that of oxidation, although they did not offer any experimental proof in support of this suggestion. Gregg [1931] in the same laboratory, found that rats on a diet of fat had an average R.Q. of 0·72.

The difficulty of interpreting the results from respiratory exchange, in man and animal, is that the process of conversion may take place in one organ, but the metabolism of the rest of the body may obscure the lowered respiratory quotient of that single organ. Therefore, it would be more significant to determine the respiratory quotient of individual tissues. Meyerhof and Lohmann [1926] obtained the respiratory quotient in serum of liver slices taken from fasting rats. They obtained quotients of 0·48, 0·76 and 0·67 for this preparation. Dickens and Šimer [1931] reported quotients of less than 0·70 for liver slices from fasting rats whether the experiments were made with phosphate or bicarbonate-buffered Ringer's solution. However, when serum was used for the determinations, an average value of 0·71 was obtained. Neither of these observers claimed a transformation of fat into carbohydrate from these low quotients. Needham observed figures as low as 0·35 for the respiratory quotient of the developing yolk sac. He was unable to detect sufficient transformation of fat into carbohydrate to explain these low quotients and concluded that they were due to some unknown retention of carbon and oxygen for synthetic purposes.

A fourth type of experimentation has been a study of the output of sugar in the urine following the injection of adrenaline into a depancreatised dog. Chaikoff and Weber [1928] obtained a definite increase of sugar by this method. They claimed that the excess sugar came from fatty acids. However, it is now well known, from the work of Cori and Cori [1928], that adrenaline mobilises muscle glycogen. Chambers *et al.* [1932] and others have demonstrated that depancreatised dogs have considerable amounts of carbohydrate in their muscles and other tissues. Therefore, the results of Chaikoff and Weber can be explained by the liberation of carbohydrate from the tissues under the action of adrenaline.

Since the majority of workers have observed a small but definite increase in the glycogen content of the liver of rats fed on fat, we thought that this problem might be solved for this particular tissue by two types of experimentation: a determination of the respiratory quotient of the fatty livers and an estimation of any change in the carbohydrate content of slices shaken in bicarbonate-Ringer for several hours.

METHODS.

Feeding of rats. The normal rats were fed on the usual laboratory diet. The rats on a fat diet were given butter, the essential salts and water. The amount of butter eaten each day varied from 3 to 7 g. After the 4th day of fat feeding, the rats were generally in poor physical condition. For this reason, the majority of experiments were made on animals that had been on butter diets for 2 or 3 days only. The rats were killed by a blow on the head. Portions of the liver were removed as quickly as possible for the glycogen determinations. The remainder of the liver was sliced for the manometric or other experiments.

Manometric methods. The method developed by Dixon and Keilin [1933] was used for the determinations of the respiratory quotient and the O_2 consumption.

Two major difficulties complicated the determination of the respiratory quotient and Q_{O_2} of the fatty livers. In the first place, it was noticed that fat separated from the tissue and collected on the sides of the vessel. Owing to this separation of fat, it was impossible to obtain the exact weight of the tissue used in each vessel. In our later experiments, this difficulty was overcome by weighing several slices of tissue before and after drying, in order to obtain the ratio for that particular liver. The slices for the manometric method were cut with a razor blade slightly moistened with Ringer's solution and were placed on a watch-glass in a covered dish which had moistened filter-paper on the sides and top until enough slices had been collected for the experiment. The slices were weighed and transferred to the vessels as quickly as possible. This gave an accurate measure of the wet weight of the tissue, and having obtained the ratio of the wet to the dry weight for that liver, the dry weight was calculated for the tissue used in the vessel of the manometer. When the calculated value of dry tissue was compared with the observed value of the tissue taken from the vessel, washed in water and dried in an oven, the calculated value was always much higher than the observed. The difference was due to the amount of substance lost by the usual procedure for obtaining the dry weight of the tissue. Since we determined the true ratio for the wet and dry tissues only in our later work, we are unable to give Q_{O_2} values for the earlier experiments.

The second major difficulty was the formation of acetoacetic acid. If aceto acetic acid is formed by the slices from a fatty liver, oxygen will be absorbed, but no corresponding amount of CO_2 will be liberated. We determined the amount of acetoacetic acid formed, using the manometric method, and we found that considerable quantities of this acid were produced in 2 hours by the fatty liver slices. A manometric method was devised by Ostern [1933] for the determination of oxaloacetic acid and it was later used by Quastel and Wheatley [1933] for their study of the production of acetoacetic acid by the liver. Krebs (unpublished experiments) has recently improved this method by using aniline citrate in place of the aniline hydrochloride. We have adopted this modification in our work. The initial value of this substance in the liver was practically zero. Liver slices from the liver of rats on normal diet produced very little acetoacetic acid. Unfortunately our observations on acetoacetic acid formation were made before we obtained a definite ratio of wet to dry tissue in each experiment. Therefore, we are unable to give absolute values of the acetoacetic formation per mg. dry tissue per hour. We can say, however, that there is a definite formation in the livers of fat-fed rats, and that this formation would affect the respiratory quotient. In the manometric method, aniline citrate was used to liberate carbon dioxide from the acetoacetic acid. This fact suggested that hydrochloric acid used in the Dixon-Keilin method might also liberate carbon dioxide in a similar way. Several experiments were made to study this reaction, using separately aniline citrate and HCl on pure solutions of acetoacetic acid. The results of one experiment are given in Fig. 1. The carbon dioxide was quantitatively liberated by HCl, as well as by the aniline citrate, but the time courses of the two processes were very different. These experiments were made at 37°. There was a delay of 15 minutes on account of the preliminary shaking to ensure thermal equilibrium. During this time, small amounts of acetoacetic acid may be broken down, especially in the presence of the citric acid, which would account for the recovery of only 80 % of the theoretical yield. The same results were obtained when similar experiments were made on normal tissue to which acetoacetic acid had been added. Thus, the routine procedure of Dixon and Keilin may be used for determining the respiratory quotient of the fatty livers provided that a sufficiently long period be allowed for the breakdown of the acetoacetic acid. This period was, in our experiments, generally about 5 hours after spilling over the hydrochloric acid.

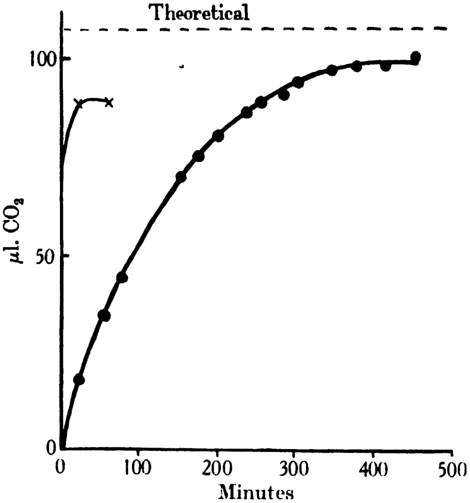

Fig. 1. Liberation of CO_2 from acetoacetic acid after adding aniline citrate and HCl. ×——× Aniline-citric acid, 80 % recovery. •——• HCl, 94 % recovery.

The remainder of the procedure for the manometric experiments was identical with the original description of Dixon and Keilin. We observed, however, that the readings, after the KOH had been admitted to the vessels, did not become constant for several hours. Elliot and Schroeder [1934] have observed similar changes, lasting for two or more hours.

Chemical methods. The Hubbard [1921, 1] method was used for the determination of total acetone bodies in the urine and the method described by Good *et al.* [1933] was used for the glycogen estimations. The glycogen estimations were made in duplicate. Only one acetone determination was made, as the amount of urine collected from a rat rarely exceeded 10 ml. for the 24-hour sample. The total fermentable carbohydrate in liver slices was determined by the method described by Cori and Cori [1933] for muscle. Estimations of β-hydroxybutyric acid in the liver slices were made by grinding the tissue in ice-cold water and carrying out the procedure described by Hubbard [1921, 2] for determining this substance in blood.

For the determination of the total fermentable carbohydrate and the β-hydroxybutyric acid in the tissue slices the following procedure was used. The tissue was cut in the same manner as for the manometric method. Alternate slices were taken for each series in order to ensure a good sampling of the liver tissue. The tissue was weighed and was placed in a small flask containing 3 ml. of bicarbonate-Ringer solution. Generally about 1·00 g. of slices was used for each determination. The solution had previously been saturated with 5 % CO_2 in oxygen. The small flask was closed with a rubber stopper through which passed two tubes, so that 5 % CO_2 in oxygen could be passed through the vessel while it was shaken in the water-bath. The gas mixture was passed through the vessels for 10 minutes. The stop-cocks were then closed and they were shaken for an additional 5 minutes before the contents of one vessel were taken for the initial value.

RESULTS.

Respiratory exchange: The results obtained on normal and fatty liver slices are given in Tables I and II and in Fig. 2. The respiratory quotients of liver slices taken from rats on a normal diet were between 0·61 and 0·87 with an average of

Table I. *Respiratory metabolism of liver tissue taken from rats on a normal diet.*

Date 1934	Weight of rat g.	Sex	Ratio of wet to dry weight of liver	Duration of exp. hours	R.Q.	Q_{O_2}	Liver glycogen %
July 4	233	M	—	1·35	0·83	—	3·09
					0·86	—	—
5	253	M	—	1·55	0·87	—	4·25
							4·45
9	398	M	—	1·38	0·82	—	4·48
					0·84	—	4·78
23	273	M	—	1·17	0·84	—	4·60
							5·07
August 2	—	M	—	1·57	0·63	—	1·56
					0·61	—	1·52
3	87	M	—	1·30	0·76	—	0·09
							0·09
7	90	M	—	1·37	0·75	—	—
					0·80	—	—
8	—	M	—	1·3	0·80	—	—
					0·76	—	—
31	276	M	—	1·5	0·72	—	3·12
					0·74	—	2·64
September 10	156	F	—	1·6	0·73	—	—
October 16	207	M	—	3·0	0·80	−7·02*	—
17	234	M	3·4	3·0	0·74	−5·46	—
18	272	M	—	3·0	0·75	−5·80*	—
22	213	M	3·5	2·0	0·83	−6·50	—
					0·83	−8·34	—

* Ratio of wet to dry tissue assumed to be 3·5.

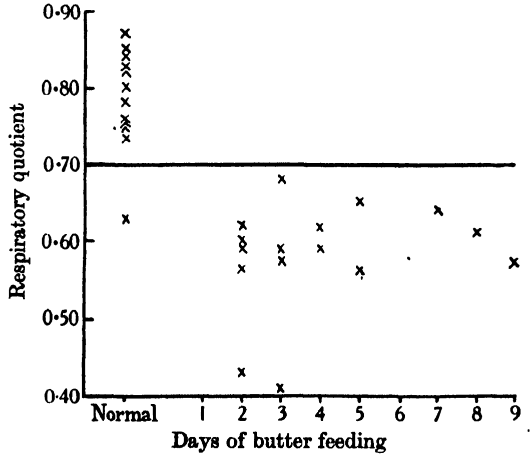

Fig. 2. Respiratory quotients of normal and fatty livers.

0·79, although only one determination, that of August 2nd, gave a value less than 0·70. Therefore, the majority of the respiration quotients for normal tissue fell within the normal limits of tissue oxidation. The range of these determinations is shown in graphic form in Fig. 2. When, on the other hand, determinations were made with the liver slices taken from fatty livers, the respiratory quotient was always below the normal level for fat oxidation, the lowest value observed was 0·41 and the highest 0·68, giving an average of 0·58. These results are given in Table II and in Fig. 2. This series of determinations on fatty livers was made after we had overcome the difficulties of obtaining accurate weights of the wet and dry tissue and was made in such a way as to eliminate the error involved in the fixation of oxygen in acetoacetic acid. The Q_{O_2} values, therefore, can be given for this series. They show some variation, not only from experiment to experiment but also in the same experiment. For example, in the experiment of November 8th, where the respiratory quotients of those determinations on the same liver were 0·55, 0·56 and 0·62, the Q_{O_2} values were 6·20, 4·13 and 7·48. However, closer agreement was obtained in other experiments.

Table II. *Respiratory metabolism of liver tissue taken from rats on a fat diet.*

Date 1934	Weight of rat g.	Sex	Days of feeding butter	Ratio of wet to dry weight of liver	Wet weight of tissue g.	Duration of exp. hours	R.Q.	Q_{O_2}	Liver glycogen %
September									
11	165	M	2	—	—	2·0	0·60	—	0·03 0·03
12	147	M	3	—	—	1·75	0·41		1·27 1·36
13	194	M	4	—	—	2·0	0·59 0·59	— —	0·65 0·52
14	178	M	5	—	—	2·03	0·60 0·72	— —	0·10 0·09
17	123	—	8	—	—	1·75	0·62	—	0·43
18	135	M	9	—	—	2·00	0·55 0·58	—	2·31 2·50
October									
24	123	M	2	2·6	0·300	2·0	0·42 0·43	−3·18 −3·70	0·39 0·42
25	101	M	3	2·5	0·300	2·0	0·58 0·59	−6·33 −5·06	0·68 0·65
26	126	—	4	2·3	0·300	2·0	0·62	−3·90	0·57 0·57
27	90	—	5	3·1	0·300	2·0	0·56	−5·00	0·56 0·54
29	96	M	7	3·1	0·300	2·0	0·68 0·60	−1·86 −3·17	0·47 0·57
31	181	M	2	3·0	0·300	2·0	0·62	−6·47	0·00 0·00
November									
1	161	M	3	2·8	0·300	2·0	0·68 0·67	3·54 −4·00	0·49 0·43
5	172	M	2	3·0	0·300	2·0	0·57 0·60	−5·88 −5·49	0·87 0·61
7	190	M	2	2·8	0·300	2·0	0·58 0·56 0·54	3·90 −4·65 −5·00	0·00 0·00 —
8	205	M	3	3·3	0·300	2·0	0·55 0·56 0·62	−6·20 −4·13 −7·48	1·52 1·57 —

A previous series of respiratory quotient determinations was made before we realised the necessity of waiting 4 or 5 hours to ensure the complete breakdown of acetoacetic acid. They fall within a somewhat lower range than those reported in Fig. 2, although the difference is not very great. This is certainly accounted for by the fact that, in this series, there was a retention of O_2 by reason of the formation of acetoacetic acid.

It is interesting to note that the ratio of wet to dry weights was different for the normal and fatty tissues. The ratio for normal tissue averaged 3·5, while that for the fatty tissue averaged 2·9.

In order to determine whether the formation of β-hydroxybutyric acid had any effect on the lowering of the respiratory quotient, two determinations of this substance were made. The results are given in Table IV. They show that the formation of 0·37 mg. β-hydroxybutyric acid per g. tissue would bind only 8 μl. O_2 per 0·3 g. of wet tissue per 2 hours. This is a negligible amount compared with the total uptake of over 1000 μl. observed in these experiments.

Table III. *β-Hydroxybutyric acid in fatty liver slices.*

Date	Days of feeding butter	Duration of exp. hours	β-Hydroxybutyric acid initial %	Increase mg. per g. of tissue	O_2 equivalent per 0·3 g. tissue per 2 hours μl.
November 19	2	3	0·0041	0·198	4·3
20	3	3	0·0214	0·37	8·0

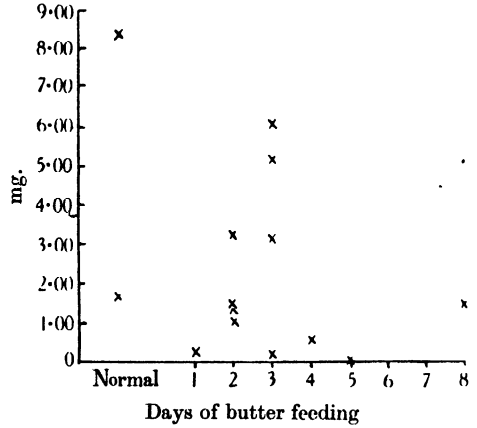

Fig. 3. Increases in total carbohydrate per g. of tissue.

Total fermentable carbohydrate. These results are given in Table IV and in Fig. 3. In order to see if comparable results could be obtained for the total fermentable carbohydrate in two series of liver slices, cut from the same liver, determinations were made on two such batches, shaken in the water-bath for the same length of time. The results of these determinations show close agreement both for the initial and final values (Table IV), illustrating that this method is adequate for determining changes in the carbohydrate content of the tissues. After these preliminary experiments, determinations were made in which one series was removed at the end of 15 minutes in the water-bath for the initial value and the other series at the end of 3 hours. Two determinations on normal

Table IV. *Total carbohydrate in normal and fatty liver slices.*

					Total carbohydrate					
					Initial		Final			
Date 1934	Sex	Weight of rat g.	Days of feeding butter	Duration of exp. hours	Fermentable %	Non-fermentable %	Fermentable %	Non-fermentable %	Increase in total fermentable carbohydrate mg. per g. tissue	Initial lactic acid mg. per g. tissue
September										
28	—	—	Normal	0	8·03	0·31	—	—	—	—
					8·12	0·33	—	—	—	—
October										
2	—	—	Normal	0	4·68	0·145	—	—	—	—
					4·47	0·24	—	—	—	—
3	—	—	Normal	2·0	—	—	3·92	0·26	—	—
							4·02	0·22	—	—
4	—	—	Normal	2·0	—	—	3·83	0·16	—	—
							3·78	0·06	—	—
5	—	—	Normal	2·0	2·48	0·09	2·64	0·20	1·60	—
6	M	150	Normal	2·0	8·72	0·29	9·55	0·00	8·30	—
8	F	95	1	3·0	0·427	0·00	0·454	0·00	0·27	—
9	F	87	2	3·0	0·457	0·00	0·585	0·00	1·28	—
10	F	94	3	3·16	1·03	0·054	1·34	0·053	3·10	—
11	F	77	4	3·0	1·19	0·00	1·24	0·00	0·50	—
12	F	89	5	3·0	3·42	0·00	3·41	0·00	−0·10	—
15	F	72	8	3·0	1·51	0·09	1·64	0·06	1·30	—
November										
10	M	261	2	3·0	0·574	0·00	0·890	0·00	3·16	—
11	M	141	3	3·06	3·12	0·08	3·63	0·00	5·10	—
12	—	234	2	3·0	1·66	0·10	1·80	0·08	1·40	—
13	M	214	3	3·5	2·53	0·16	3·13	0·05	6·00	0·64
14	—	300	2	3·0	1·38	0·00	1·48	0·00	1·00	1·14
15	—	314	3	3·0	1·38	0·00	1·39	0·00	0·10	1·00

tissue gave an increase in carbohydrate during this period of shaking. These results are in contrast to those of Takane [1926]. Takane found a decrease in total carbohydrate in normal tissue and only observed an increase in the presence of added lactate. We determined the lactic acid content of the tissue at the beginning of three experiments (Table IV). The maximum initial lactic acid content was 1·14 mg. per g. tissue. If all the lactic acid were converted into fermentable sugar, it would account for a rise of the same amount. We did not determine the content of lactic acid at the end of our experiments. If lactic acid had disappeared, there would be no evidence that it had passed into carbohydrate and thus no correction could be applied to the carbohydrate increase. We then turned our attention to the livers of fat-fed animals. Again, an increase in carbohydrate was observed. The most marked increase was obtained on November 13th, where the increment was 6 mg. per g. tissue. In this experiment, the initial lactic acid value was only 0·64 mg. per g. tissue. Therefore the conversion of lactic acid into carbohydrate could account for only a small proportion of the total increase. In a few of our experiments the carbohydrate increase could be explained by assuming that all the initial lactic acid was converted into fermentable carbohydrate, as in the experiment of November 14th. The majority of our experiments gave increases greater than 1 mg. per g. wet tissue, therefore the increment of carbohydrate must have come from some other source. Taken in conjunction with the low respiratory quotients, we regard this as most important evidence for the formation of carbohydrate from fat.

Glycogen. The changes in the liver glycogen of the rats on a butter diet are given in Fig. 4. These results are stated in terms of g. glucose per 100 g. wet tissue. The values of the glycogen content of livers from rats on a normal diet varied between 1·52 % and 5·25 % with one exception. This value was very low, 0·10 %. On the diet of butter, the liver glycogen practically disappears on the 1st day and then gradually returns, reaching an average value of about 1 %. A high value was obtained for the liver glycogen of a rat fed for 9 days on butter. The averages for our 7, 8 and 9-day experiments are not as conclusive as for the 2nd, 3rd and 4th days, owing to the smaller number of experiments made on the longer periods of fat feeding. Our results show an increase of glycogen under

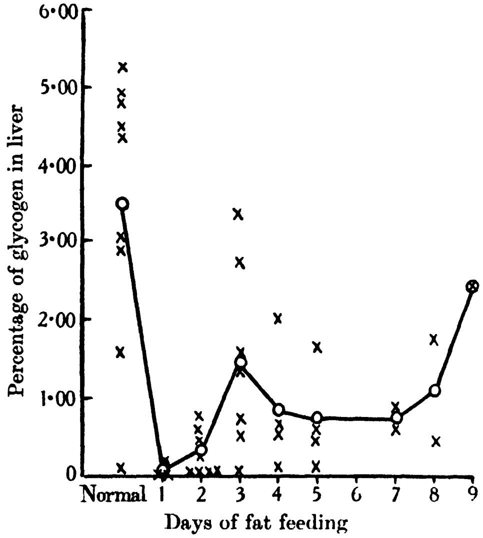

Fig. 4. Variation of liver glycogen on normal and fat diets. The circle gives the average value in each case.

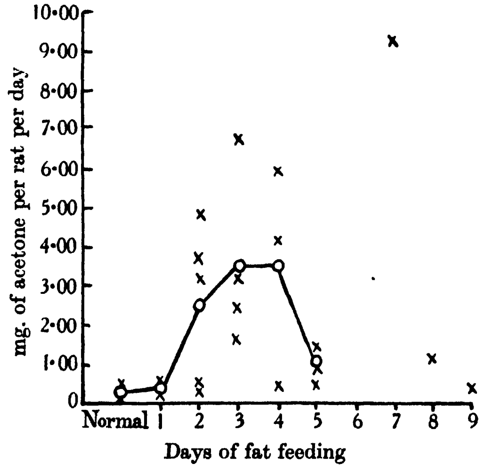

Fig. 5. Total acetone bodies excreted per rat per 24 hours.

these conditions. In one experiment, we kept a rat on butter diet for 7 days and then on the normal diet for 2 days. This rat gave an exceptionally high value for liver glycogen, 9·5 %.

Total acetone bodies. The results are given in Fig. 5 in terms of mg. acetone per rat excreted in the urine during the 24 hours before the animal was killed. There is a slight correlation between the appearance of glycogen in the liver and the disappearance of the total acetone bodies from the urine. The results in both series are so scattered that it is difficult to draw any conclusions from the averages. In general the acetone bodies showed an initial rise, starting on the 2nd day, reaching a peak on the 3rd and 4th days and then falling again, although the one experiment on the 7th day gave a very high value. Wigglesworth [1924] has observed similar changes in the acetone bodies of rats on a diet of butter.

An attempt was made to determine the changes in the fat content and the iodine number of liver slices in order to see if any increase in desaturation or if any decrease in fat content could be detected during a 3-hour period of metabolism. The results were so variable for two series of slices shaken for the same period of time that this procedure was discontinued.

Discussion.

The experiments described in this paper offer good evidence in favour of the hypothesis that fat can be converted into carbohydrate in the liver of the rat. In view, however, of the sharp controversy which the question has raised in the past, we are anxious to define as precisely as possible exactly what we believe our experiments to have proved. We have shown that, on a fat diet but not on a normal one, the respiratory quotient of rat liver slices *in vitro* is well below 0·7. Therefore there is a causal connection between fat feeding and low respiratory quotients. Further, we have shown that carbohydrate can be synthesised by liver slices *in vitro*. The matter may, perhaps, be put into more general terms, as follows. The respiratory quotient in our experiments is a function of the fat diet: carbohydrate synthesis also takes place under these conditions. Since we have eliminated by direct experiment many of the fallacies which surround the determination of the respiratory quotient, we feel that there is strong presumptive evidence that the low quotients do, in fact, indicate carbohydrate synthesis; this presumption is greatly strengthened when we find such a synthesis actually occurring. We should feel that neither finding, taken by itself, would be very conclusive; on the other hand, both taken together, represent a strong case.

One or two further points merit attention. In the livers of two rats on a normal diet, we found carbohydrate synthesis, and in one case this synthesis was larger than any found in the livers of the fat-fed animals. If gluconeogenesis were a process occurring only in animals fed on an entirely abnormal diet of pure fat, it would probably be of little importance. It has, on the contrary, been assumed by many authors, without experimental proof, to occur in normal animals. The R.Q. of the normal liver slices may be assumed to have been in the neighbourhood of 0·79, the average figure for normal livers, and one which does not indicate the formation of carbohydrate from fat. We have, in fact, no evidence to show that the carbohydrate was not formed in these cases from protein. Supposing, however, that it was formed, in whole or in part, from fat, it is quite possible that in a carbohydrate-rich liver the process would result in a lowering of the R.Q. from unity to a level which was still well above that of fat oxidation. On the other hand, in carbohydrate-poor livers, such as are those on the 2nd and

3rd days of a fat diet, the liver is probably oxidising fat, and carbohydrate synthesis would lower the R.Q., starting from a base line, not of 1·0, but of 0·7. We have calculated, in fact, that the synthesis of 3·0 mg. carbohydrate per g. liver slices would lower the R.Q. from 0·79 to 0·63 in our respiratory experiments. Although some of our experiments showed a synthesis of carbohydrate of less than 3·0 mg. per g. tissue, in four experiments the carbohydrate synthesis was greater than 3·0 mg. Similarly, in a number of experiments, the R.Q. was less than 0·63. Our average value was in fact 0·58.

The increase in liver glycogen in the rats fed on butter occupies an intermediate position between the results of those observers who have reported large increases and those who have found very small changes. The averages for our determinations for each day show a decided increase on the 3rd day in contrast to the absence of glycogen on the 1st and 2nd days. Without our other data, it might be assumed that this increase is due to the glycerol fraction of the fat, an assumption made by Gregg [1931] to explain similar results. With the lowered respiratory quotient and the determination of an actual increase in carbohydrate in liver slices the more probable explanation for this rise is that part of the carbohydrate is coming from fat.

SUMMARY.

1. The respiratory quotient of slices of liver from a rat fed on a normal diet averages 0·79, while that from a rat fed on butter averages 0·58.

2. The carbohydrate content of liver slices taken from a liver of a rat fed on butter shows a definite increase after 3 hours' shaking in bicarbonate-Ringer medium at 37°.

3. The glycogen content of the liver of rats fed on butter falls to practically zero on the 1st day of this diet. It gradually rises reaching a level of almost 1·00 % on the 4th and 5th days of butter feeding.

4. The acetone bodies in the urine of rats increase, reach a maximum on the 3rd or 4th day of butter feeding and then decrease.

5. The lowering of the respiratory quotient and the increase in carbohydrate of the liver slices indicate that conversion of fat into carbohydrate takes place in the liver of a rat fed on butter. The same conversion may take place in a normal liver.

REFERENCES.

Bodey, Lewis and Huber (1927). *J. Biol. Chem.* **75**, 715.
Burn and Ling (1929). *Quart. J. Phar. Pharm.* **2**, 1.
—— and Marks (1926). *J. Physiol.* **61**, 497.
Chaikoff and Weber (1928). *J. Biol. Chem.* **76**, 813.
Chambers, Kennard, Pollack and Dann (1932). *J. Biol. Chem.* **97**, 525.
Cori and Cori (1928). *J. Biol. Chem.* **79**, 321.
—— —— (1933). *J. Biol. Chem.* **100**, 323.
Dickens and Šimer (1931). *Biochem. J.* **25**, 985.
Dixon and Keilin (1933). *Biochem. J.* **27**, 86.
Elliot and Schroeder (1934). *Biochem. J.* **28**, 1920.
Good, Kramer and Somogyi (1933). *J. Biol. Chem.* **100**, 485.
Gregg (1931). *J. Nutrition*, **4**, 385.
—— (1933). *Amer. J. Physiol.* **103**, 79.
Greisheimer (1931). *J. Nutrition*, **4**, 411.

Hawley, Johnson and Mulin (1933). *J. Nutrition*, **6**, 523.
Hubbard (1921, 1). *J. Biol. Chem.* **49**, 357.
—— (1921, 2). *J. Biol. Chem.* **49**, 375.
Magnüsson (1929). *Skand. Arch. Physiol.* **55**, 278.
Meyerhof and Lohmann (1926). *Biochem. Z.* **171**, 381.
Ostern (1933). *Z. physiol. Chem.* **218**, 160.
Page and Young (1932). *Biochem. J.* **26**, 1528.
Quastel and Wheatley (1933). *Biochem. J.* **27**, 1753.
Soskin (1929). *Biochem. J.* **23**, 1385.
Takane (1926). *Biochem. Z.* **171**, 403.
Takao (1926). *Biochem. Z.* **172**, 272.
Wigglesworth (1924). *Biochem. J.* **18**, 1203.

XLII. PROTEIN AND THE DIETARY PRODUCTION OF FATTY LIVERS.

By HAROLD JOHN CHANNON and HARRY WILKINSON.

From the Department of Biochemistry, The University, Liverpool.

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The observation by Best *et al.* [1932] that fatty livers could be produced in rats which received a diet consisting of mixed grain with 40 % of beef fat provided a means whereby the curious action of choline in preventing and curing such dietary fatty livers has been extensively studied [Best and Huntsman, 1935]. On a number of occasions however use of this diet has failed to produce in our rats fatty livers of an intensity similar to that recorded by the Toronto workers, while its deficient nature may cause weight losses in the experimental animals. Since the amount of liver fat is changed by starvation, it may also be influenced by this type of undernutrition and complicating factors in the interpretation of results may thereby be introduced.

A governing factor in fatty liver production on diets containing 40 % of fat has been shown to be the choline content [Best and Huntsman, 1935]. With this finding in mind an attempt was next made to produce dietary fatty livers in rats under conditions in which no loss of body weight occurred. For this purpose a diet of caseinogen 20, starch 30, beef fat 40, salt mixture 5, marmite 5 and vitamins A and D was given. Both the caseinogen and starch had been exhaustively extracted by alcohol and ether and save for the small amount of choline present in the marmite—1·75 mg. per rat per day—this diet was choline-free. Fatty livers did not result. This amount of choline is of no importance since the mixed grain diet of Best, Hershey and Huntsman [1932] which causes fatty livers in rats contains enough choline to give each animal about 8 mg. per day. This failure suggested that some factor other than the choline content was influencing the production of fatty livers on diets containing 40 % of fat, and consideration of various results in the literature suggested the level of protein intake as the most probable. In this connection the wide variations in the relative amounts of glyceride and cholesteryl ester appearing in the "cholesterol" fatty liver caused by feeding diets containing 1–2 % of cholesterol were considered. Calculation of the approximate compositions of the diets used by different workers [Okey, 1933, 1, 2; Chanutin and Ludewig, 1933; Best *et al.*, 1934; Channon and Wilkinson, 1934] indicated that the protein content of the diets might be a factor in the control of the amount of liver glyceride. Accordingly the effect of varying the protein content of the diet at the expense of the carbohydrate, with a constant fat content (40 %) and an adequate supply of vitamins and salts, has been studied. The object was to evolve a diet which would cause fatty livers without loss of body weight if possible and to study the part played by protein in fatty liver production. Further, in order to study any effect of protein in controlling the fatty liver produced by feeding rats on pure sucrose [Best and Huntsman, 1935], two further diets were included; these were fat-free and contained 5 and 30 % of protein respectively with 85 and 60 % of glucose hydrate. All the diets used have been substantially choline-free.

These experiments have been used at the same time to attempt to elucidate a further point in fatty liver production. Great variations in the percentage of liver fat among individual rats under the same fatty liver-producing dietary regime often occur and the extent of these variations can be readily seen by reference to the paper of Best and Huntsman [1935] where variations from 5·5 to 26·6 % among individuals from one experiment are recorded. This degree of variation must have some significance. The frequency of its occurrence is such as to make the use of large numbers of animals necessary, apart from rendering interpretation of results somewhat uncertain. The degree of fat infiltration occurring in the liver in starvation in rats and rabbits has been shown to be related to the amount of fat present in the depôts [Dible, 1932; Dible and Libman, 1934]. It was possible that the variations in the amount of fat in the fatty livers of rats on a high fat diet were related to the amount of depôt fat. This has been investigated by a study of the fat content of the liver and carcass of each individual animal in these experiments.

EXPERIMENTAL.

The general plan of the experiment was to feed groups of rats on various diets for 21 days. The animals were then killed by a blow on the head 12 hours after the last meal. The livers were removed and weighed for analysis, the gastro-intestinal tracts removed and the carcasses stored in a refrigerator until they could be analysed.

The diets used are described in Table I. The caseinogen was B.D.H. "fat-free casein" which had been further extracted three times with boiling industrial alcohol. The choline content of the constituents of the diets was determined by assay on the isolated rabbit intestine after hydrolysis and acetylation. Save for the marmite, the constituents were choline-free. The marmite contained 350 mg. of choline per 100 g. and since it constituted 5 % of all the diets, each animal in the different groups ingested about 1·5 mg. of choline per day. The average actual amounts of choline ingested by the animals in each group are recorded in Table II.

Table I. *Description of the diets.*

Group ...	A	B	C	D	E	F	G	H
Diet								
Caseinogen	0	5	10	20	30	50	5	30
Beef dripping	40	40	40	40	40	40	0	0
Glucose hydrate	50	45	40	30	20	0	85	60
Marmite	5	5	5	5	5	5	5	5
Salt mixture	5	5	5	5	5	5	5	5
Cod-liver oil	One drop per rat every 3 days							

The fat content of the individual livers was determined by ethereal extraction after acidification of the livers following saponification with aqueous NaOH. After evaporation to dryness of the washed ethereal extract the weight of the "fat" was obtained. Separation of this material into its constituent fatty acids and unsaponifiable matter was not carried out because experiments showed that the combined weights of unsaponifiable matter and fatty acids obtained by further alcoholic saponification were in all cases 98 % of that of the original material. The fat content of the carcasses was determined either by a similar method or by saponification of the alcoholic extract of the minced carcass. In the latter cases the minced carcasses were treated many times with successive volumes of boiling alcohol and the combined extracts saponified and treated as already described.

Results.

In Table II are recorded data concerning the number of rats used in each group, their changes in weight during the experiment, the average food intake per rat per day, the caloric value of this food and its choline content.

Table II. *Weight records of the animals and the food intake.*

Group	A	B	C	D	E	F	G	H
Protein % of diet ...	0	5	10	20	30	50	5	30
No. of animals ...	6	6	6	6	6	5	6	6
Av. weight at beginning of experiment (g.)	141	143	141	136	139	148	141	136
Av. weight at end of experiment (g.)	122	147	153	179	182	186	140	146
Gain or loss (g.)	−19	+4	+12	+43	+43	+38	−1	+10
Av. food intake per rat per day (g.)	8·3	9·2	8·0	9·0	9·0	8·5	11·7	13·5
Caloric intake per rat per day (Cals.)	48·0	53·4	46·4	52·5	52·5	50·6	44·5	51·1
Choline intake per rat per day (mg.)	1·46	1·70	1·42	1·59	1·59	1·50	2·07	2·38

Diets A to F contained 40 % fat; G and H were fat-free.

In Table III the percentages of "fat" (total fatty acids and unsaponifiable matter) present in the livers and the carcasses of the individual animals are set out.

Table III. *The percentage of "fat" in the livers and carcasses.*

(g./100 g.)

Group ...	A		B		C		D	
	liver	carcass	liver	carcass	liver	carcass	liver	carcass
	9·93	10·30	22·83	16·13	6·74	17·12	6·55	—
	7·35	8·92	8·53	19·20	10·35	18·94	4·48	12·04
	7·59	17·44	15·83	18·00	7·19	14·21	7·91	12·27
	8·55	11·25	7·68	20·16	6·68	13·54	4·94	—
	10·54	15·08	7·67	16·16	7·70	—	7·41	
	9·71	11·84	12·40	15·94	5·78	—	7·75	16·78
Mean	8·95	12·40	12·49	17·60	7·35	—	6·50	—

Group ...	E		F		G		H	
	liver	carcass	liver	carcass	liver	carcass	liver	carcass
	4·54	—	—	—	3·07	7·81	4·79	17·06
	6·05	15·39	4·21	11·14	3·42	8·42	4·46	8·13
	6·73	19·15	5·56	15·61	3·57	7·16	4·20	16·57
	6·21	—	6·18	15·57	5·01	9·74	4·84	14·16
	7·01	14·34	6·67	17·60	6·88	11·64	4·67	—
	6·20	23·02	5·40	17·73	6·01	13·94	4·85	4·68
Mean	6·12	—	5·60	15·53	4·66	9·78	4·64	—

DISCUSSION.

1. *Diets (B–F) containing 40 % of fat with varying percentages of protein.* The wide variations in the percentages of fat in the individual livers are seen in Table III. For discussion purposes the mean values are recorded in that table, although the limitations caused by the relatively small numbers of animals are recognised. The average figures for the "fat" content of the livers of the animals receiving 5, 10, 20, 30 and 50 % of protein are 12·49, 7·35, 6·50, 6·12

and 5·60 % respectively. These diets were substantially choline-free, for the choline intake of each rat in the different groups varied only between 1·42 and 1·70 mg. per day. As mentioned in the introduction, this amount of choline is considered to be of no significance in the interpretation of the results and further, since it is virtually constant at 1·5 mg. per rat per day, any possible effect is the same for all the groups. It is clear therefore that the amount of protein in the diet is a factor controlling the amount of fat appearing in the liver irrespective of any effect of choline, the degree of fat infiltration increasing with decreasing protein content. The 12·49 % of fat in the livers of animals which received 5 % of protein is similar to the figure often recorded by the Toronto workers as occurring in rats receiving the mixed grain diet with 40 % of fat. Such a diet would on our calculations contain 6 % of protein. The decreasing amount of fat appearing in the liver with increasing protein intake explains also our previous failures to produce fatty livers in rats on diets of high fat content but choline-free, because in all these cases protein constituted at least 20 % of the diet. These investigations further provide a method for producing fatty livers in rats by dietary means on diets free from choline without the possibility of complications due to weight loss. Thus the diet containing 5 % of protein and 40 % of fat resulted in the livers of the animals containing 12·49 % of fat at the end of 3 weeks with an average weight increase of 4 g. per animal.

This control of the glyceride content of the liver by the protein content of the diet has been further confirmed in experiments on the "cholesterol" fatty liver. We administered to one group of rats a diet containing caseinogen 5, fat 20, glucose 65, marmite 5, salt mixture 5, vitamins A and D, with 2 % cholesterol. To a second group the same diet was given save that the protein content was raised to 30 % at the expense of the sugar, which was reduced to 40 %. After 3 weeks the animals were killed and their livers analysed. The results obtained are recorded in Table IV.

Table IV. *The liver lipoids of rats fed on a diet containing 20 % fat and 2 % cholesterol, but free from choline.*

(g./100 g. liver.)

Group ...	J	K
Percentage protein in diet	5	30
Phosphatide	1·79	2·54
Glyceride	25·42	5·54
Free cholesterol	0·294	0·452
Cholesteryl oleate	4·42	7·91

The figures in Table IV appear amply to confirm the finding of the control of glyceride deposition in the liver by the level of the protein intake, for whereas the glyceride content of the livers of animals receiving 5 % of protein (group J) is 25·42 %, that of group K, which received 30 % of protein, is only 5·54 %. It is of interest to note that the amount of cholesteryl esters is some 80 % higher in group K, compared with group J, but at this stage no interpretation of this finding can be provided.

These results show therefore that in the "cholesterol" fatty liver the relative amounts of glyceride and cholesteryl esters depend in the case of choline-free diets on the protein content of the diet, the glyceride fraction being far more extensively involved and in the reverse direction. They explain the varying degree to which these two constituents occur in the livers of rats fed on diets containing cholesterol as reported by different workers, a question which has been discussed in a previous paper [Channon and Wilkinson, 1934]. A number of other points arise from consideration of Table IV and other data concerning this experiment: (*a*) The total fatty acid content of the livers of group J (protein 5 %, fat 20 % with cholesterol 2 %) is 27·5 %. On the other hand that of group B in Table III (protein 5 %, fat 40 % without added cholesterol) is 12·49 %, a figure half as great. This increase does not seem to be related in any way to the cholesteryl ester content of the livers, because the livers of group K in Table IV contained 80 % more cholesteryl esters than those of group J. (*b*) In group J the phosphatide percentage, 1·79 %, is the lowest which we have yet encountered in any group of fatty livers, just as the "fat" content is the highest. (*c*) The liver weights expressed as a percentage of the body weights were, in group J 5·55 % and in group K 3·54 %; the former figure again is the highest we have encountered and is to be compared with the normal figure of about 3 %. (*d*) The normal iodine value of rat liver fatty acids is about 115. Those of group J (total liver fatty acids 27·5 %) had an iodine value of 78·7, while in group K (total liver fatty acids 10·52 %) the iodine value was 80·7. These figures illustrate the fact that under the conditions of these experiments the iodine value does not fall in proportion to the degree of fat infiltration. Thus in group J where the livers contained about nine times the normal amount of fatty acids, the iodine value has fallen only from 115 to 78·7, while in group K (liver acids three times the normal amount) the iodine value was 80·7. Admixture of one part of the original liver acids (I. V. 115) with 8 parts of those of the food (I. V. 40) would yield a mixture of acids having iodine value 48. Clearly therefore the liver acids of group J have not been derived by direct absorption of the mixed food acids. Further they contained 29·5% of solid acids of iodine value 13 (Twitchell) a percentage differing little from the normal. Whether the unsaturated liver acids, which in group J amount to 1·58 g. per animal, of iodine value 106, have been derived by preferential absorption of the unsaturated acids of the food or depôt fats, cannot be determined. More detailed evidence along these lines may however throw light on any possible desaturation process.

The general finding that the protein content of the diet controls the degree of glyceride infiltration of the liver may have a number of explanations, of which mention will be made of one only. In view of the known action of choline and of betaine in preventing fat infiltration [Best and Huntsman, 1932] it may imply that certain amino-acids may be converted into these substances in the tissues. Best and Huntsman [1935] draw attention to the fact that Engeland [1909] suggested that betaines might arise in the tissues by methylation of amino-acids. It is possible that a number of other compounds of types similar to betaine and choline, which might have physiological actions in controlling the liver fat level, may arise from different amino-acids, although at the present time choline and betaine themselves are alone known to be active. Thus choline itself might be derived from glycine, as was suggested by Rosenfeld [1930] as a result of his study of the synthesis of lecithin by chickens. Another possible precursor of choline might be serine. It is of interest however that the protein used in our experiments, caseinogen, contains very little glycine or serine. We are therefore continuing these studies by producing fatty livers in rats on a diet containing 5 % caseinogen and are adding to the diets supplements of these and other amino-acids. In a previous paper [Aylward *et al.*, 1935] studies were made of the changes in the lipoids of the livers of rats following a fat- and cholesterol-containing meal free from and with added choline. The diets used contained 20 % of caseinogen and the decrease in liver phosphatide at the seventh hour in the livers of the animals receiving the choline-free diet was much greater than on the diet which contained choline. If this finding be confirmed, it would imply that the action of protein in preventing fat infiltration was much slower than that of choline itself and may suggest that the conversion of some precursor into a substance having the same action as choline is being relatively slowly effected.

A further point of interest is that although the percentage of liver fat decreases with increase of the protein percentage in the diet, even in group F (protein 50 %) it is still 5·66 %, a figure which is to be regarded as about 1 % higher than the normal.

It will be observed from Table III that in the animals of group A, which received no protein in the diet, the livers contained 8·95 % of fat, *i.e.* substantially less than did those of rats which received 5 % of protein. We do not propose to discuss this figure because the weight losses of the animals, an average of 19 g. in 21 days, are too great to permit of any deductions being made with certainty.

With the results recorded in Table III are to be compared those of Best *et al.* [1935]. These workers obtained fatty livers in rats receiving a diet of 15 % of protein and 20 % of fat for 21 days (average figure 10·5 %). According to our results such a diet should not produce fatty livers, for the figures in Table III suggest that, if 15 % of protein were present in the diet with as much as 40 % of fat, the fat content of the liver would be about 7 %, whereas the fat content of their diet was only 20 %. It is not unlikely that these discrepant findings are to be ascribed to the weight losses of the animals of Best *et al.*, which were 9 g. per animal in a period of 21 days, the diet used being deficient in vitamins. The experiments of Best and Ridout [1933] in which rats received a grain diet with 20 % of fat did not result in fatty livers. Since the protein content of this diet would be only 8 %, it might have been expected from our data that fatty livers should have resulted, even though the fat content of the diet was only 20 %. These particular results cannot be compared with our own, however, because the average weight loss of the animals was 23 g. in 31 days, while the diet was such that each animal received about 8 mg. of choline per day, facts which complicate any comparison.

2. *Diets (G–H) containing no fat with 5 % and 30 % of protein.* No difference in the fat contents of the livers of these groups occurred, nor did fatty livers result, the average value being 4·65 %. From the point of view of future experiments it should be pointed out that the individual variation among the livers of the animals which received 30 % of protein was very much less than in any of the groups in this experiment. This suggests such a diet to be very suitable as a preparatory one for ensuring the greatest possible constancy of the fat content of the liver before further dietary treatment.

Best and Huntsman [1935] found that normal animals receiving exclusively sucrose developed fatty livers. The present results show that a diet of 5 % protein and glucose does not cause fatty livers. We think the explanation of these differences is again to be found in complicating factors of weight loss, for whereas our animals had an average weight loss of 1 g. in 21 days, the rate of weight loss of those of Best and Huntsman was rapid.

3. *The relationship between the amount of depôt fat and the amount of liver fat.* The figures recorded in Table III show that no relationship exists between the amount of depôt fat and that of liver fat. If the ratios of the percentage of the carcass fat to that of the liver fat be calculated for the animals which received a 40 % fat diet with 0, 5, 10, 30 and 50 % of protein, the average figures re-

sulting are 1·38, 1·40, 2·17, 2·94 and 2·77; if the individuals of one particular group are considered (group B, 40 % fat, 5 % protein) the ratios of carcass fat to liver fat are 0·70, 2·25, 1·14, 2·62, 2·11, 1·28. It is clear therefore that these results provide no evidence for the view that fat infiltration in the liver under these particular dietary conditions is influenced by the amount of depôt fat, and in this they are to be contrasted with the starvation experiments referred to in the introduction. This question of individual variation must be of significance and needs further investigation.

SUMMARY.

1. Groups of rats have been fed on complete diets containing 40 % fat with varying amounts of protein (from 5 to 50 %) for a period of 3 weeks and their livers and carcasses analysed for fat content. The diets were substantially choline-free, but each rat actually received 1·5 mg. of choline per day, which was present in the yeast extract used to supply vitamin B complex.

2. The amount of fat appearing in the liver was conditioned by the amount of protein in the diet irrespective of any action of choline.

3. The diet containing 40 % of fat and 5 % of protein is adequate for weight maintenance and for the production of livers containing 12·49 % of fat in 3 weeks.

4. This control of the glyceride content of the "fat" fatty liver has been confirmed by experiments on the production of the "cholesterol" fatty liver, for livers of animals receiving a diet containing 5 % of protein, 20 % of fat and 2 % of cholesterol contained at the end of 3 weeks 25·42 % glyceride and 4·42 % cholesteryl esters. On the other hand the livers of another group receiving the same amounts of fat and cholesterol but with 30 % protein, contained 5·54 % glyceride and 7·91 % cholesteryl esters.

5. The possibility of the action of the protein in controlling liver fat being due to its providing amino-acid precursors which are converted either into choline or betaine or substances of similar physiological action is discussed.

6. No relationship exists between the amount of fat infiltration in the liver and the amount of the depôt fat.

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REFERENCES.

Aylward, Channon and Wilkinson (1935). *Biochem. J.* **29**, 169.
Best, Channon and Ridout (1934). *J. Physiol.* **81**, 409.
—— Hershey and Huntsman (1932). *J. Physiol.* **75**, 56.
—— and Huntsman (1932). *J. Physiol.* **75**, 405.
—— —— (1935). *J. Physiol.* In press.
—— MacLean and Ridout (1935). *J. Physiol.* In press.
—— and Ridout (1933). *J. Physiol.* **78**, 415.
Channon and Wilkinson (1934). *Biochem. J.* **28**, 2026.
Chanutin and Ludewig (1933). *J. Biol. Chem.* **102**, 57.
Dible (1932). *J. Path. Bact.* **35**, 451.
—— and Libman (1934). *J. Path. Bact.* **38**, 269.
Engeland (1909). *Ber. deutsch. chem. Ges.* **42**, 2962.
Okey (1933, 1). *J. Biol. Chem.* **100**, lxxv.
—— (1933, 2). *Proc. Soc. Exp. Biol. Med.* **30**, 1003.
Rosenfeld (1930). *Biochem. Z.* **218**, 36.

XLIII. A STUDY OF CYSTINURIA IN TWO SISTERS.

BY EVELYN MARION HICKMANS AND
WILLIAM CAREY SMALLWOOD.

From the Children's Hospital, Birmingham.

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THE subjects of this investigation are sisters, one of whom, the younger, was first referred to the Birmingham Children's Hospital by Dr Burns of Redditch for the investigation of albuminuria. She was admitted and on examination was found to have cystinuria. A subsequent examination of a specimen of urine from the sister revealed cystine crystals and she, also, was admitted for investigation and observations on metabolism.

Family history.

Cystinuria is one of a group of disorders which have been aptly described by Sir Archibald Garrod in the picturesque phrase "inborn errors of metabolism". An enquiry was instituted, therefore, into the occurrence of the derangement in other members of the family, and with the co-operation of the patients' doctor and parents many specimens of urine were collected from each of seventeen blood relatives, at intervals of several weeks, and examined by us for cystine. The tests applied were:

(1) Microscopical examination of sediment: (*a*) from the fresh specimen, (*b*) after acidifying with dilute acetic acid and standing for one week, and (*c*) from acidified specimens concentrated by evaporation.

(2) The Sullivan reaction as modified by Brand *et al.* [1930].

(3) The cyanide-nitroprusside reaction described by the same authors.

These investigations revealed well-marked cystinuria in one other relative, whose position in the genealogical tree is shown in Fig. 1. The positions of the two subjects of this investigation are indicated by the encircled figures, while crosses denote the existence of members who were not examined. In addition to the discovery of one other definite cystinuric it was found that the urine of several other members of the family gave faintly positive results with one or other of the chemical tests for cystine, and a few showed one or two hexagonal crystals after prolonged microscopical examination of an acidified specimen of urine concentrated by evaporation.

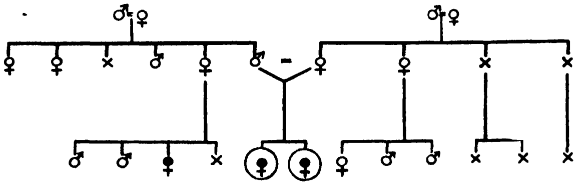

Fig. 1. Genealogical tree.

♂ ♀ male and female normals respectively; **♀** female cystinurics.

These findings, together with the results of tests for albumin and estimations of total amino-acids, appear in Table I. It will be seen that the presence of traces of cystine in the urine of one individual was not constant and that specimens in which, on microscopy, two or three hexagonal crystals were found did not always yield positive chemical tests and *vice versa*.

Various writers [Loewy and Neuberg, 1907; Lewis and Simon, 1902] have suggested that the degree of cystinuria may vary considerably from time to time and even disappear temporarily. At first we were tempted to believe that these other members of the family were occasional or mild cystinurics and that the union of two such individuals had resulted in two florid examples of the metabolic error. We do not feel justified, however, in advancing this thesis on such slender evidence. There was no consanguinity of parentage in any of the families and no family history of stone or of nephritis known to the parents of the two children investigated.

Clinical history.

W.B. Aged 12 years. First child in family of two. Admitted to Birmingham Children's Hospital on April 26th, 1932.

History. Quite well until 6 months previously; since then vaguely unwell; easily tired, frequently complaining of backache referred to both loins and relieved by lying down. For 3 months prior to admission scalding micturition but no increased frequency of micturition or other urinary symptoms. Occasional headaches for many years.

Examination. A tall slender girl: weight 5 stone 6 pounds: nutrition moderately good and general appearance one of health. Mentality normal. No abnormality of cardiovascular, respiratory, alimentary or nervous systems. B.P. 125/80. Kidneys neither enlarged nor tender.

Urine. S.G. 1030. Reaction slightly alkaline to litmus. Albumin: light cloud. Sugar: nil.

Deposit. Cystine crystals in large numbers in specimens which were allowed to stand, both before and after acidifying with acetic acid. Sullivan and nitroprusside tests both strongly positive for cystine.

X-ray of renal tract—no evidence of stone.

Blood.	Urea	(fasting)	30·9	mg./100 ml.
	Non-protein-nitrogen	,,	30·3	,,
	Uric acid	,,	2·81	,,
	Amino-acid-nitrogen	,,	11·48	,,
	Chlorides	,,	459	,,
	Cholesterol	,,	182	,,
	Creatinine	,,	1·26	,,

Urea concentration test (*Calvert's*) showed normal renal function:

15 g. urea given.
Specimen A. Volume 500 ml.: Urea 2·32 g./100 ml.
Specimen B. Volume 930 ml.: Urea 0·39 g./100 ml.

J.B. Aged 10 years. Second child in family. Admitted to Birmingham Children's Hospital, April 6th, 1932.

History. Always a delicate child though never suffering from any definite disease of any system. One year before admission commenced to complain of backache, was easily tired and had frequent frontal headaches. These symptoms continued. In November 1931 the child was noticed to be passing very little urine, was seen by Dr Burns of Redditch and found to have albuminuria. No history of haematuria or other urinary symptoms. She was thought to be abnormally thirsty at this time. Twelve months before admission the child had an acute febrile illness in which she vomited and developed a scarlatiniform rash but never peeled. The albuminuria persisted and the child was sent to hospital for investigation of renal function.

Examination. A tall slender girl—weight 4 stone 4 pounds—of healthy appearance, normal mentality and moderately good nutrition. Cardiovascular, respiratory and nervous systems normal. Many carious teeth present: alimentary tract otherwise normal. Kidneys neither enlarged nor tender.

Table I. *Investigation of cystinuria in blood relatives of patients.*

	Hexagonal crystals in the urine			Sullivan's test*		Nitroprusside test*		Amino acid-N* mg./100 ml.		
	1st specimen	2nd specimen	3rd specimen	2nd specimen	3rd specimen	2nd specimen	3rd specimen	2nd specimen	3rd specimen	Albumin
Relatives on Mother's side:										
Mother, B. B.	2 or 3 crystals	–	–	+	+	–	+	2·8	6·4	Sl. haze
Grandfather, J. A.	–	–	–	–	+	–	–	10·5	9·4	Sl. haze
Grandmother, M. A.	–	–	–	+	+	–	+	10·0	11·7	–
Aunt, G. T.		2 crystals		–		–		6·0		–
Cousin, B. T.		–		–		–		8·75		–
Cousin, I. T.		–		–		+				–
Cousin, F. T.		–		–		+		12·2		–
Relatives on Father's side:										
Father, T. B.	–	–	–	–	+	–	–	14·5	14·4	–
Grandfather	–	–	–	–	+	–	+	13·5	12·7	Sl. haze
Grandmother	–	–	–	–	+	–	–	11·6	15·5	–
Aunt, Al. B.		3 crystals	–	–	–		–		15·5	–
Aunt, An. B.		–	–	+	+	–	+	7·24	11·7	–
Uncle, E. B.	–	–	–	–	–	–	–		8·7	–
Aunt, M. S.		4 crystals	–	–	–	+	+		8·7	–
Cousin, J. S.		–	–	+	–		–		8·7	–
Cousin, K. S.		3 crystals	–	+	+	+	+		14·7	–
Cousin, M. S.		Many crystals	Many crystals	+ + +	+ + +	+ + +	+ + +		22·2	Haze

* 1st specimen not tested. + denotes a trace colorimetrically. + + + denotes a definitely positive test.

Urine. On admission S.G. 1020. Reaction very slightly alkaline to litmus. Albumin: heavy haze. Sugar: nil.

Deposit. Cystine crystals in large numbers. Sullivan and nitroprusside tests for cystine both strongly positive.

X-ray of renal tract—repeated several times. On each occasion much colon gas was present but no stone was seen.

Blood. Wassermann reaction negative.

Urea	(fasting)	36·8	mg./100 ml.
Non-protein-nitrogen	,,	36·5	,,
Uric acid	,,	2·15	,,
Amino-acid-nitrogen	,,	6·18	,,
Cholesterol	,,	170	,,
Chlorides	,,	482	,,
Creatinine	,,	1·27	,,

A twenty-four hour specimen of urine—volume 373 ml.—collected when the child was having ordinary hospital diet showed:

Urea	1·72	g./100 ml.
Uric acid	0·051	,,
Creatinine	0·098	,,
Total nitrogen	1·60	,,
Chlorides	0·61	,
Amino-acid-nitrogen	0·0297	,,
p_H	7·05	,,

Silver articles placed in contact with the skin of both children for periods of five days showed no discoloration, an observation which suggests that sulphur compounds were absent from the sweat of our patients. The urine of both children developed a greenish colour and gave off hydrogen sulphide and ammonia on standing.

Presence in the urine of tyrosine, leucine, putrescine and cadaverine.

The urine of both patients was examined repeatedly for the presence of leucine and tyrosine and for the diamines cadaverine and putrescine. Specimens were concentrated by evaporation *in vacuo* and the following tests performed:

Examination of the concentrate for crystals of leucine and tyrosine.

Tests with Millon's reagent.

Treatment of the concentrate with β-naphthalenesulphonyl chloride (method of Abderhalden and Shittenhelm [1905]).

Benzoylation method of Baumann and Udransky [1889].

Phenyl isocyanate method of Loewy and Neuberg [1904].

These tests failed to reveal the above amino-acids and diamines.

Test for the presence of a precursor of cystine in fresh specimens of urine.

A perusal of the literature on cystinuria reveals frequent references to the presence in the urine of cystine precursors, which usually break down quite rapidly with the liberation of free cystine, especially after the addition of dilute acetic acid. These may, however, from time to time prove more stable and resist the action of the somewhat hypothetical urinary ferments which are supposed to bring about the necessary chemical changes. Wallis and Garrod [quoted by Garrod, 1923] reported a case in which cystine could not be found by the Gaskell method, and which showed cystine crystals in the urine only after six months' standing. Garrod remarks "the inference cannot be avoided that under certain conditions cystinurics excrete a sulphur compound which is not free cystine but a compound or unoxidised derivative thereof".

From time to time various workers [Klemperer and Jacoby, 1915; Looney *et al.*, 1923] have reported a diminution in the cystine excretion (as determined colorimetrically) after the prolonged administration of alkali without significant changes in the neutral sulphur values, and these findings have been regarded as further evidence in favour of the excretion in freshly voided urine of a cystine complex and as an explanation of the phenomenon of intermittent cystinuria. This subject receives careful consideration by Brand *et al.* [1930] who, in their observations on a cystinuric, found that the freshly voided urine of their patient gave a negative Sullivan reaction, but after standing for 24 hours the brilliant red colour indicating a positive reaction had developed and this colour continued to increase in intensity for a further 24 hours before fading. In a similar experiment we have observed an almost exactly similar phenomenon in both of our patients. The red colour commenced to develop at the end of the first hour, deepened gradually during the next 48 hours and then slowly faded.

Metabolism experiments.

The source of the cystine excreted in the urine, whether of endogenous or exogenous origin, and the effect of diet on the amount of this excretion have been the points studied by various workers. The investigations here recorded were undertaken in order to test the effect of various diets on the excretion of urinary cystine, in an endeavour to discover a diet sufficient for the requirements of these growing children which was least productive of cystinuria and therefore less likely to lead to stone formation; simultaneously an attempt was made to locate the source of the cystine.

The feeding experiments carried out were as follows:

(1) Ordinary hospital diet.
(2) Ordinary hospital diet *plus* pure cystine.
(3) Low cystine, medium protein diet.
(4) High cystine, medium protein diet.
(5) High cystine, high protein diet.
(6) Ordinary hospital diet *plus* pure cystine for estimation of faecal loss of cystine.

Method. The experiments were carried out concurrently on the two patients and on a normal control—a boy aged 12 years, who had recovered from pneumonia a few weeks earlier and appeared to be in good health at the time of the investigation. Throughout the experimental period all these children were allowed to play in the ward and garden each day. All urine passed was collected in 24-hour specimens under toluene and subjected to the following estimations. Total nitrogen (micro-Kjeldahl method using Koch and McMeekin's modification of Nessler's solution, as quoted by Peters and Van Slyke [1932], ammonia (Folin's aeration method), urea (urease), uric acid [Folin, 1930], creatinine [Folin, 1914], amino-acid-nitrogen [Folin, 1922], cystine (Looney's method using Folin's [1930] uric acid reagents), sulphur in all forms [Fiske, 1921] and p_{H} (electrometrically, using the quinhydrone electrode or by indicators).

A summary of the results obtained is shown in Figs. 3, 4 and 5.

When this work was completed and the figures for the various periods analysed, it was found that there were well-marked daily variations in the output of various urinary constituents, particularly in total nitrogen, uric acid, creatinine, cystine and neutral sulphur. These diurnal variations were all in the same direction so that the graphs for each showed great similarity. Further, these daily variations coincided in the three children, suggesting that the same factor was operating in all three, and as this could not be of a dietetic nature it was thought to be the result of changes in metabolism associated with differing amounts of exercise. Consequently we readmitted the younger child, J.B., for investigation of this point.

Effect of exercise. The patient was given a generous varied diet containing similar foodstuffs in the same amounts each day and supplying approximately 86 g. of protein and 2000 Calories daily. In addition to fluids taken with meals she was given 4 pints of lemonade daily, to ensure a diuresis and consequent rapid elimination of metabolic products.

The metabolism period commenced at 9 a.m. after a preliminary period of 21 hours in bed on the diet. During the first 48 hours the child remained at rest in bed. For the first 9 hours of the next 24-hour period she played around the ward without indulging in any strenuous games (indicated in Fig. 2 as "light exercise"). On the following day she was made to run around the garden and to skip until tired (indicated as "moderate exercise"). After a further period of complete rest in bed she was again exercised and the metabolism period was completed with 48 hours in bed. Fig. 2 shows the excretion of various waste products during this 8-day period. The first 24 hours yielded high total nitrogen and low sulphur values, and we regret that the child was not given a longer time to become stabilised before the urine was collected. The urea-nitrogen figures remained at a very constant level over the whole period, indicating the constant nitrogen intake. Light exercise was associated with increased excretion of cystine and neutral sulphur but did not affect the uric acid or creatinine figures. Moderate exercise resulted in a greatly increased excretion of uric acid, creatinine, cystine and neutral sulphur, so that the graphs for the output of these various bodies are almost identical. The figures for total nitrogen tend to show similar variations. The increased uric acid and creatinine excretions must result from an

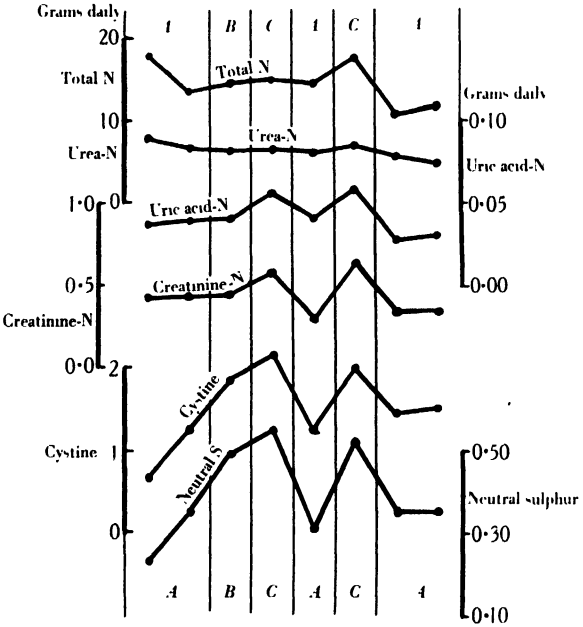

Fig. 2. J.B. Analyses of urine to demonstrate the effect of exercise.

A, at rest in bed; *B*, light exercise; *C*, moderate exercise.

increased tissue metabolism associated with exercise and it seems highly probable that the increased cystine excretion was also of endogenous origin. In the investigation of a case of cystinuria, Brand *et al.* [1930] noted an increased creatinine excretion on the day when their patient had 2 hours' exercise (handball) and mentioned that Alsberg and Folin [1905] also discovered an increased excretion of creatinine associated with exercise. They did not comment on the fact that associated with this increased creatinine excretion there was a well-marked increase in the output of cystine in their patient. Robson [1929] also noted well-marked daily fluctuations in another case, in spite of careful control of the protein intake, which he was unable to explain. He did not mention whether his patient was in bed or not throughout the metabolism period and it may be that exercise was the factor responsible for his results.

The importance of the exercise factor was not appreciated at the time when our feeding experiments were carried out and the children were not confined to bed. The exercise taken, although varying a little from day to day, was never strenuous and, in our opinion, was not sufficient to affect seriously the average figures for a 5-day period.

Feeding experiments.

Experiment 1. Hospital diet with a medium protein and low cystine content. After a preliminary period of 2 weeks on this diet the metabolism of nitrogen and sulphur was followed over a 5-day period and it was then noticed that the albuminuria which was present in both cystinurics on admission to Hospital had almost disappeared. The cystine excretion at this time was low and a remarkable feature of the investigation was the increased albuminuria which occurred later when the patients were having a high protein diet and the output of cystine was increased. This phenomenon was observed in both children, the degree of albuminuria reflecting quite accurately the degree of cystinuria. The control child was always albumin-free. Orthostatic tests carried out on the cystinurics were negative.

Another striking feature was the high p_H of the urine of both cystinurics throughout the whole of the metabolism period. The 24-hour specimens of urine were collected under toluene, kept under exactly the same conditions, and the p_H was estimated at the earliest possible opportunity. The figures for the control child varied between 5·2 and 7·3, with an average of about 6 for the whole period, and the ammonia values were low. The urines of the cystinurics were much more alkaline, occasionally reaching figures as high as p_H 9. The highest figures corresponded with the peaks in the graph for ammonium compounds, although even when the ammonia values were low and corresponded with those of the control child, p_H values of between 7 and 8 were frequently found; further, the alkalinity of any specimen from the cystinurics increased considerably on standing, although fresh specimens of urine examined from time to time showed this same tendency to alkalinity. We are unable fully to explain this phenomenon. The children were observed over a long period of time, their urinary tracts appeared to be free from infection, they were not given medicines or other preparations which might have contained alkalis, and the experiment was adequately controlled. This peculiarity may have been of very great practical importance in preventing calculus formation in the two children.

The excretions of nitrogen and urea are very similar in the cystinurics and the control. The sisters show high amino-acid-nitrogen and neutral sulphur values, as would be expected.

Experiment 2. Hospital diet plus *cystine.* To Diet No. 1 pure cystine (prepared from animal hair and supplied by B.D.H. Ltd.) was added in the following amounts:

1st day	1 g.	14 g.
2nd day	2 g.	
3rd day	4 g.	
4th day	7 g.	

On the 5th, 6th and 7th days no cystine was added.

The average figures for the first 5 days of this period, described here as the "cystine ingestion period", together with those for the following 2 days when the children were again on hospital diet only, are shown in the figures. In the case of W.B. (Fig. 4) the output of cystine was raised very slightly during the ingestion period and still higher figures were found on the 2 subsequent days, but this small increase is probably of no significance, especially as the figures for neutral sulphur were not increased. The output of inorganic sulphur was doubled during the ingestion period but returned to a level lower than the average for that period on the 2 following days. A calculation shows that about 60 % of the added cystine was metabolised during the 7 days. The fate of the residual 40 % formed

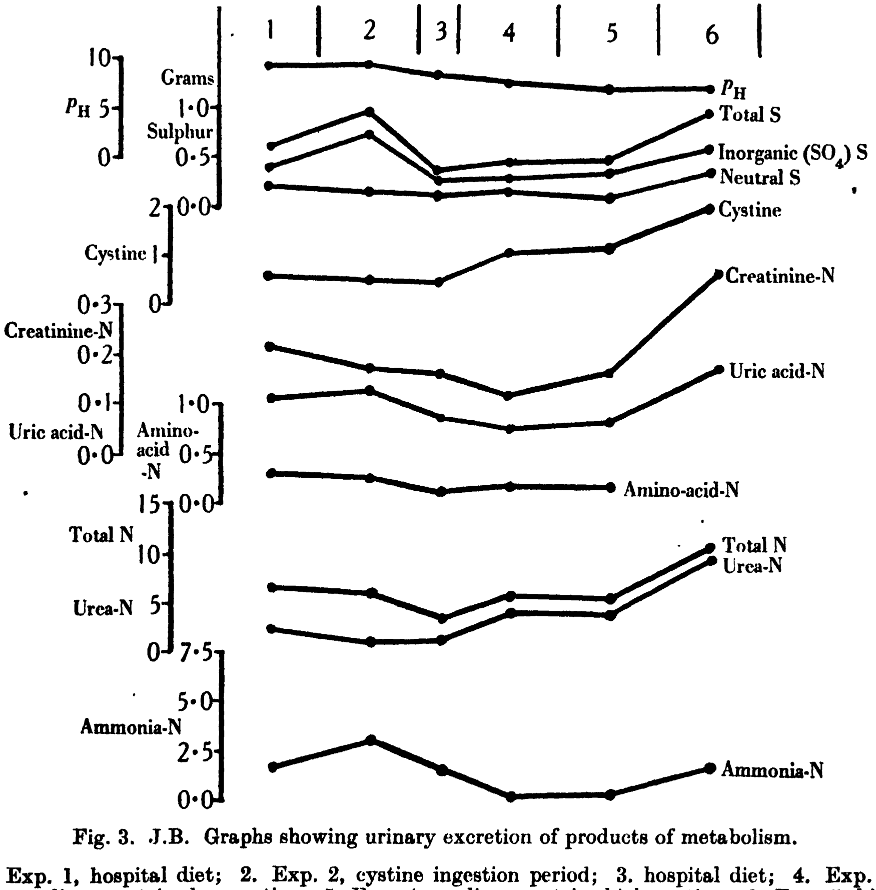

Fig. 3. J.B. Graphs showing urinary excretion of products of metabolism.

1. Exp. 1, hospital diet; 2. Exp. 2, cystine ingestion period; 3. hospital diet; 4. Exp. 3, medium protein, low cystine; 5. Exp. 4, medium protein, high cystine; 6. Exp. 5, high protein, high cystine.

the object of an experiment carried out when all the dietetic investigations were completed, but which may conveniently be mentioned here. W.B. was given the weighed hospital diet and after a period of several days on this regime, in which to become stabilised, 4 g. of cystine were given by mouth. The increased output in the faeces, estimated colorimetrically, amounted to 1·43 g., representing 36 % of that ingested. It would appear, therefore, that cystine given as such was oxidised quantitatively to inorganic sulphate and that the mechanism of oxidation was not at fault.

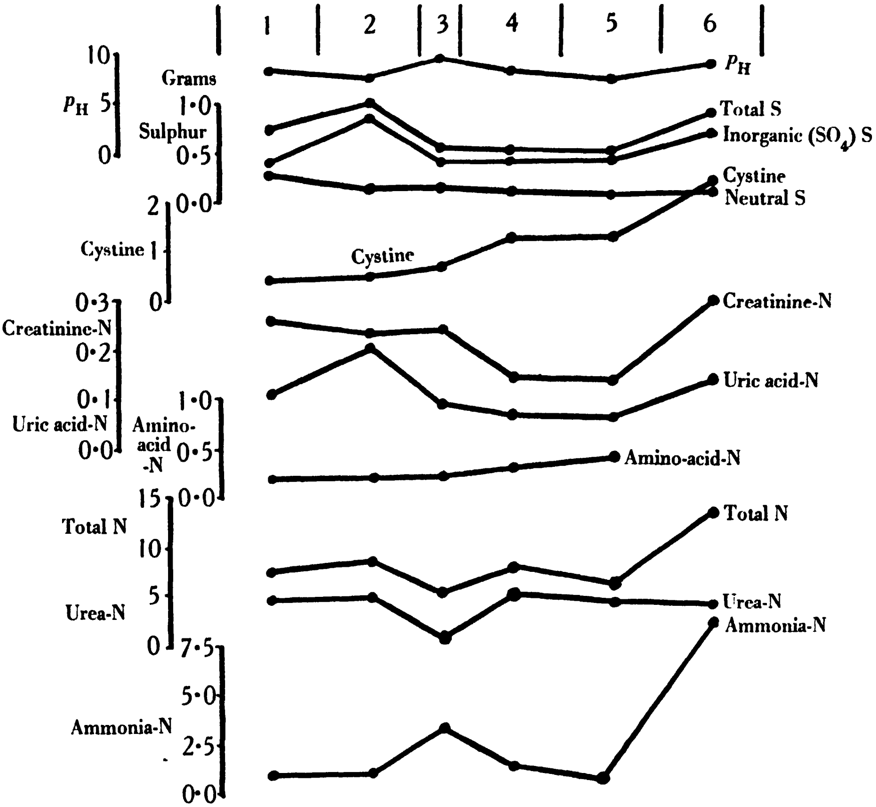

Fig. 4. W.B. Graphs showing urinary excretion of products of metabolism 1, 2, 3, *etc.* as in Fig. 3.

In the case of J.B. (Fig. 3) the output of cystine was slightly less than in Exp. No. 1 and only 47 % of the amount ingested was metabolised. The control (Fig. 5) oxidised 77 % of his ingested cystine and in addition showed a slight increase in neutral sulphur excretion. On 2 days his urine gave a slight coloration with the nitroprusside test for cystine.

Robson [1929], in a similar investigation of a male cystinuric aged 23 years, who was given 20 g. of cystine by mouth over a period of 4 days, found that 85 % of the ingested cystine was broken down and excreted as inorganic sulphate. The absence of any increase in urinary cystine during the ingestion period is in accord with the findings of most other investigators. Lewis and Lough [1929] calculated that the cystinuric investigated by them was able to oxidise about

64 % of the pure cystine ingested, the remainder being lost by failure of absorption or by destruction in the bowel by micro-organisms. Our results demonstrate the failure of absorption from the bowel but do not suggest any breakdown of cystine by micro-organisms.

Experiment 3. Low cystine, medium protein diet. After a few days' return to hospital diet the patients were given one consisting of one pound of apples and three pints of milk daily. This provides 5·8 g. protein, 0·6 g. sulphur (including, at most, 0·40 g. cystine) and 1390 Calories. After 3 preliminary days on the diet urine was collected for a further period of 5 days. On this low cystine but medium protein intake the output of cystine was considerably raised in both cystinurics, resulting in a negative cystine balance of 0·8 g. per day in W.B. and 0·6 g. per day in J.B. Our results are in accord with those of Lewis and Lough [1929] who conducted a similar experiment, and additional evidence is furnished of the endogenous origin of a significant portion of the cystine excreted.

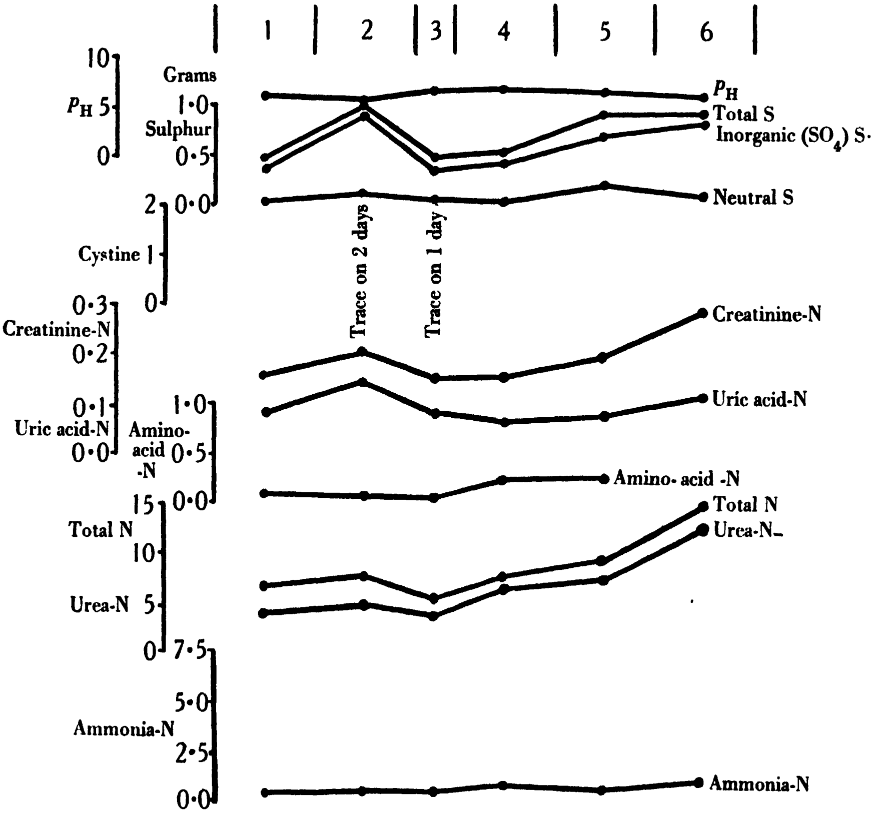

Fig. 5. Control. Graphs showing urinary excretion of products of metabolism. 1, 2, 3, *etc.* as in Fig. 3.

In the case of W.B. there was a lessened excretion of total and neutral sulphur but approximately the same output of inorganic sulphate as on the basal diet. J.B. did not appear to absorb the milk protein as well as that of a mixed diet, indicated by a lessened urinary nitrogen output. At the same time poor absorption of protein cystine resulted in a diminished inorganic and total sulphur excretion. On this diet, therefore, both patients appeared to be excreting very largely endogenous cystine, the low cystine intake being reflected in the low sulphur output.

That the excretion of endogenous cystine was stimulated by increased protein intake is confirmed by the results of feeding with a high protein diet.

Experiment 4. High cystine, medium protein diet. This consisted of one pint of milk, four eggs, half-a-pound of apples, four ounces of brown bread and one ounce of butter daily, and provided 53 g. of protein, 1271 Calories and 0·76 g. of sulphur—mostly cystine sulphur. The protein content was very similar to that of the previous diets. The average figures for the 5-day period show neither increased amino-acid nor cystine output. The control appeared to metabolise the added sulphur to inorganic sulphate, but either the cystinurics failed to absorb the extra cystine of the sulphur molecule, as the amino-acid itself or as some precursor, or, alternatively, they absorbed and stored it. In any case, the cystine output was not affected by the cystine intake.

The fall in the figures for uric acid and creatinine in Exps. 3 and 4 may be explained as the result of a vegetarian diet.

Experiment 5. The protein intake was doubled by adding eight ounces of cooked lean meat to Diet No. 4, the cystine content remaining practically at the same level. The cystine output was doubled in the two sisters and at the same time more sulphur was oxidised, as indicated by the rise in total and inorganic sulphur values. These rises were associated with similar increases in the figures for total nitrogen, urea, uric acid and creatinine. The results of this experiment are in conformity with those obtained by Robson [1929], who found that on a high protein diet the increased elimination of cystine was in almost direct proportion to the increased nitrogen excretion. We are unable to calculate the cystine intake on this diet, but it seems highly probable that there was again a negative cystine balance and that the output was of endogenous origin and increased by a high protein diet. Lewis and Lough [1929] reached a similar conclusion in a comparison of the effects of diets on varying cystine and protein values; these workers found that the excretion of cystine varied with the total nitrogen excretion and did not appear to be related to the cystine content of the diet.

It is generally recognised that diets rich in animal protein have a decided effect in stimulating tissue metabolism and raising the metabolic rate: this effect is thought to be due to the action of certain of the amino-acids resulting from such protein digestion which stimulate cellular activity. Further evidence on this point is furnished by our cases which show increases in uric acid and creatinine excretion more than twice as high as those obtained on the previous diet (No. 4) of half the protein value and therefore, partially at least, of endogenous origin, the result of increased tissue breakdown. The figures for neutral sulphur in the child W.B. showed no rise corresponding to the increase in the figures for cystine as estimated colorimetrically—in fact the graph for neutral sulphur throughout the metabolism period shows a steady fall. In the case of J.B. the graphs for neutral sulphur and cystine correspond more closely. The cystine-sulphur excretion as calculated from the cystine (estimated colorimetrically) was, on the whole, higher than the total neutral sulphur; an observation made by many previous workers, each of whom concluded that the colorimetric estimation tends to give unduly high readings. Estimation of cystine in the previous 24 hours' urine was carried out each day at approximately the same time; hence, at the time of the examination, the breaking down of any cystine precursors present in the freshly voided specimens should have proceeded to about the same extent. We therefore regard these figures as a more reliable indication of cystine excretion than those for neutral sulphur, even though they may give inaccurate absolute values. The neutral sulphur figures for W.B. are probably inaccurate.

The undetermined nitrogen varied throughout the metabolism periods. Figures as high as 33 % were reached by W.B., 25 % by J.B. and 29 % by the control, and over the whole period the undetermined nitrogen was somewhat higher in the control child than in either cystinuric.

DISCUSSION.

The subjects of this investigation show many interesting features in common. Both were admitted with albuminuria which was not of the orthostatic type or associated with clinical evidence of renal insufficiency or other evidence of kidney damage, and which increased or decreased (to the point of disappearing) with increased and diminished output of urinary cystine. These observations are of great interest and may be of importance in view of the work of Cox *et al.* [1929], Newburgh *et al.* [1925], and Curtis and Newburgh [1926; 1927], who fed young rats on synthetic diets containing free cystine and produced in their animals an acute nephritis from which many of them died. Newburgh and Curtis [1927], by feeding rats on diets rich in caseinogen, beef protein and lactalbumin, were able to demonstrate the markedly nephropathogenic nature of the lactalbumin—a protein with a very high cystine content (4 %).

Abderhalden [1903] described an infant, one of a family of cystinurics, who died of malnutrition at the age of 21 months. At autopsy the internal organs were found to contain crystalline deposits of cystine visible to the naked eye and in many ways similar to the deposits of sodium biurate in gout. The same writer [1922] observed kidney damage in rats ingesting free cystine and tyrosine and was able to demonstrate crystals of these amino-acids in the kidney tubules. It is difficult to reconcile these animal experiments with the results of feeding cystine to normal adults and to cases of cystinuria. Nevertheless, it seems probable that, in our patients, the excretion of the greater quantities of this abnormal urinary constituent, possibly associated with deposition of crystals in the renal tubules, was associated with some renal damage and a leak of protein into the kidney tubules. The urinary cystine in our patients we believe to be entirely endogenous. The exercise experiment demonstrates the importance of this factor in affecting the cystine output. Feeding isolated cystine showed that the amino-acid is completely oxidised to inorganic sulphate, even when administered in large quantities, a finding which is in agreement with those of other writers [Hele, 1909; Looney *et al.*, 1923; Robson, 1929]. Feeding with a diet which contained a high content of cystine sulphur in the natural foodstuff did not result in any greater output of cystine than when the children were given one with a low cystine value and similar protein content, but there is no evidence that the extra cystine of the diet was absorbed or in any way metabolised. We submit that the increased excretion of urinary cystine on a diet high in animal protein is of endogenous origin, the result of increased tissue metabolism due to the specific dynamic action of that protein and not derived from the cystine of the ingested protein molecule. The practical importance of this observation is obvious and our patients were sent home on a diet of low protein content, which they had taken conscientiously until they were seen again recently—two years after the original investigations were carried out. Both children were apparently quite well and free from any symptoms referable to the urinary tract. There was no evidence of stone formation or urinary infection.

With the co-operation of the parents, 24-hour specimens of urine were collected from each of the two girls when they were at home taking their usual diet and indulging in their normal activities. That from the elder child, W.B., was free from albumin and contained 9·45 g. of total nitrogen and 0·585 g. of cystine (colorimetric). The specimen from J.B. showed a faint haze of albumin (far less than when first seen two years previously), 5·1 g. of total nitrogen and 0·442 g. of cystine—all of them reasonably low figures.

The high p_H of the urine, which formed such a notable feature of this investigation, has already received consideration, and in view of this finding it was considered unnecessary to try the effect of administration of alkalis. For this reason also, many specimens of urine had first to be acidified with dilute acetic acid and allowed to stand before cystine crystals appeared, and the greater solubility of cystine in an alkaline solution may explain the fact that the subjects of this study, although passing relatively large amounts of the amino-acid, remain free from calculus formation.

SUMMARY.

Two cases of cystinuria occurring in sisters are recorded, together with the results of the examination for cystinuria in other members of the family.

With the aid of the Sullivan reaction, evidence is presented which indicates that in these patients a precursor of cystine was excreted into the urine, which later decomposed with the liberation of free cystine.

Other abnormal constituents—tyrosine, leucine, putrescine and cadaverine—were absent from the urine of these subjects.

Both passed urine with an abnormally high p_H and it is suggested that this may explain their freedom from stone.

Evidence is brought forward to show that the excretion of large amounts of cystine may bring about a certain amount of kidney damage, producing an albuminuria which varies with the amount of cystine excreted.

Cystine prepared from hair when administered by mouth did not produce an increased excretion of cystine in the urine but was oxidised quantitatively to sulphates. Much of it was unabsorbed and excreted in the stool.

Foodstuffs with a high cystine content did not cause increased cystinuria; the extra cystine may have remained unabsorbed or have been absorbed and stored but was neither oxidised to sulphate nor excreted in the urine as such.

The endogenous origin of some of the excreted cystine is demonstrated, and the effect of a high animal protein diet in increasing the cystinuria is explained as the result of a stimulation of tissue metabolism with liberation of more endogenous cystine.

We suggest that cases of cystinuria should be given a diet containing only small amounts of animal protein.

It is a pleasure to acknowledge the debt of gratitude we owe to Miss Eva L. Tonks for her help in carrying out much of the laboratory work recorded in this paper, to Prof. L. G. Parsons for his stimulating interest in the work and permission to record the investigation of his cases and to the Medical Research Council for a personal grant to one of us (E.M.H.), and a further grant towards defraying the cost of the investigation.

REFERENCES.

Abderhalden (1903). *Z. physiol. Chem.* **38**, 557.
—— (1922). *Pflüger's Arch.* **195**, 204.
—— and Shittenhelm (1905). *Z. physiol. Chem.* **45**, 468.
Alsberg and Folin (1905). *Amer. J. Physiol.* **14**, 54.
Baumann and Udransky (1889). *Z. physiol. Chem.* **13**, 562.
Brand, Harris and Biloon (1930). *J. Biol. Chem.* **86**, 315.
Cox, Smythe and Fishback (1929). *J. Biol. Chem.* **82**, 95.
Curtis and Newburgh (1926). *J. Clinic. Invest.* **2**, 611.
—— —— (1927). *Arch. Int. Med.* **39**, 817.
Fiske (1921). *J. Biol. Chem.* **47**, 59.
Folin (1914). *J. Biol. Chem.* **17**, 469.
—— (1922). *J. Biol. Chem.* **51**, 393.
—— (1930). *J. Biol. Chem.* **86**, 179.
Garrod (1923). Inborn errors of metabolism. (Oxford Medical Publication.)
Hele (1909). *J. Physiol.* **39**, 52.
Klemperer and Jacoby (1915). *Arch. Int. Med.* **16**, 503.
Lewis and Simon (1902). *Amer. J. Med. Sci.* **123**, 838.
—— and Lough (1929). *J. Biol. Chem.* **81**, 285.
Loewy and Neuberg (1904). *Z. physiol. Chem.* **43**, 355.
—— —— (1907). *Biochem. Z.* **2**, 438.
Looney, Berglund and Graves (1923). *J. Biol. Chem.* **57**, 515.
Newburgh and Curtis (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 963.
—— Marsh, Clarkson and Curtis (1925). *J. Amer. Med. Assoc.* **85**, 1703.
Peters and Van Slyke (1932). Quantitative clinical chemistry. (Baillière, Tindall and Cox, London.)
Robson (1929). *Biochem. J.* **23**, 138.

XLIV. SOME BIOLOGICAL PROPERTIES OF EQUILIN.

BY KÁROLY DAVID AND SAMUEL ELZEVIER DE JONGH.
From the Pharmaco-Therapeutic Laboratory of the University of Amsterdam.

(Received December 19th, 1934.)

EQUILIN, an important member of the large group of oestrogenic substances discovered in recent years, is chemically related to menformon (oestrone).

Equilin was originally prepared from the urine of pregnant mares by Girard [1932] and co-workers; the empirical formula ($C_{18}H_{20}O_2$) had 2 hydrogen atoms less than that of oestrone; it had M.P. 238–240° (corr.) as against 255·5–256·5° for oestrone, and $[\alpha]_D^{20^\circ}$ was +308° (in dioxan) as against +163°. We obtained very similar figures for our own preparations, *viz.* M.P. 236–237·5° uncorr. and $[\alpha]_D + 307^\circ$ in dioxan.

The biological examination of equilin is justified by the fact that it constitutes the chief impurity even of crystalline commercial oestrone. For comparison of the various biological actions of pure equilin with pure oestrone equal quantities of both have been investigated in comparative assays. By equal quantities we mean not identical weights but quantities which produce the same oestrogenic effect in mice, this animal being the one with which our routine estimations are always performed. The following comparative assays were made: (*a*) oestrus in the castrated rat, (*b*) genital growth in the young female rat, (*c*) opening of the vagina in the young rat, (*d*) "antimasculine" action in the rat, (*e*) "paradoxical" effect in the castrated rat (growth of seminal vesicles), (*f*) nipple growth in the adult male guinea-pig, (*g*) epithelial metaplasia in the genitalia of the male castrated mouse.

We only determined which of the two substances under consideration would produce the stronger reaction per given number of mouse units. To establish a quantitative ratio in each case would have rendered the investigation too laborious and was not attempted.

We first determined as nearly as possible the exact dose of equilin required to induce oestrus in the castrated mouse, injecting oestrone 3 × 0·1 ml. into one group and equilin into another group of mice and next week performing a cross-test on the same subjects (summary in Table I). The letters *a*–*g* represent graded types of vaginal smear, progressing in alphabetic sequence towards oestrus, *a*–*d* being negative, *e*–*g* positive types [de Jongh *et al.*, 1932]. The oestrone used in our experiments was tested on several hundred animals and found to be equal in oestrogenic potency to the International standard preparation. Accordingly it contains one I.U. in 0·1 γ whether comparison is made in rats or in mice. This

Table I. *Number of animals and stage of cycle achieved or surpassed.*

		$\geqslant a$	b	c	d	e	f	g	Positive
I.	0·1γ oestrone	21	21	21	21	21	18	5	21/21 = 100 %
	0·12γ equilin	21	21	21	17	16	12	6	16/21 = 76 %
				Also 0·12γ equilin <0·1γ oestrone					
II.	0·1γ oestrone	48	46	45	43	42	33	15	42/48 = 87·5 %
	0·15γ equilin	48	46	44	41	37	36	22	37/48 = 77·1 %
				0·15γ equilin <0·1γ oestrone					
III.	0·1γ oestrone	24	24	20	20	19	13	6	19/24 = 79 %
	0·18γ equilin	24	24	24	24	23	16	10	23/24 = 96 %
				0·18γ equilin >0·1γ oestrone					

international unit of oestrone will as a rule yield positive smears in 70 % of our animals when given in oil solution (3 injections of 0·1 ml. within 36 hours).

Evidently from Table I the dose of equilin equivalent to 1 international unit in mice is not less than 0·15γ. A similar effective dose-ratio was obtained when equilin and oestrone were given to mice by mouth (Table I *a*), the actual quantities being 1·5γ equilin and 1·0γ oestrone in 3 equal portions of oil solution.

Table I *a*.

	⩾*a*	*b*	*c*	*d*	*e*	*f*	*g*	Positive
1·5γ equilin	23	18	13	12	12	8	3	12/23 = 52 %
1·0γ oestrone	23	18	13	13	12	10	3	12/23 = 52 %

In subsequent experiments oestrone and equilin were therefore given in the ratio of 2 : 3 by weight.

(*a*) *Oestrus in rats.*

1γ oestrone and 1·5γ equilin were given in 3 subcutaneous injections; 26 rats were used and cross-tested with the following result:

	⩾*a*	*b*	*c*	*d*	*e*	*f*	*g*
Oestrone	26	21	16	14	8	6	3
Equilin	26	24	23	22	20	20	18

Clearly the action of equilin was stronger than could have been anticipated from its effect on mice, and this was confirmed by a further experiment on 53 rats using half the dose of equilin and the full dose of oestrone.

	⩾*a*	*b*	*c*	*d*	*e*	*f*	*g*
Oestrone	53	46	34	33	28	25	16
Equiline	53	50	41	39	30	27	15

In the rat equilin is four-thirds as oestrogenic per mg. as oestrone, which does not agree with Girard's [1933] finding of only one-seventh.

The I.U. of equilin, when tested in mice, is 0·15 γ, while when tested in rats it is 0·075 γ. From these data we draw the important conclusion that it is not sufficient to describe a preparation as containing a certain number of I.U. without reference to the kind of animals used for the testing. For more intensive discussion of this question see Laqueur [1935].

(*b*) *Growth of genitalia.*

Small doses (0·05γ oestrone and 0·075γ equilin) in 0·1 ml. were given twice daily for 14 days (May 29th–June 12th) for fine differentiation of the relative activities. Rats from 3 litters were castrated and equally divided between the

Table II.

	Weight (g.)									
Rat	Before	After		Uterus mg.		Vagina mg.		Preputial glands mg.		Substance
3807	35	55	51	85	66	98	115	17	22	Equilin
3808	27	47		59		116		16		
3809	34	50		66		145		19		
3810	37	41		59		110		32		
3811	37	63		63		106		24		
3812	31	53	72	45	45	97	93	19	28	Oestrone
3813	28	67		44		89		22		
3814	39	86		52		94		28		
3815	36	70		48		98		24		
3816	36	83		38		86		45		
3817	26	43	56	33	30	73	69	16	19	Oil
3818	31	48		24		62		12		
3819	36	76		32		78		25		
3820	35	50		32		61		20		
3821	36	53		30		71		24		

two groups. Increase in body weight was very irregular despite the use of litter-mates.

The increases in weight of the uterus and vagina were greater, though the average body weight was lower, in the equilin group. The weight of the vagina is less significant, because less accurately determinable, than that of the uterus. The action of equilin on the preputial glands is negligible, that of oestrone apparently stronger, largely on account of the exceptional reaction of animal 3816. The confirmatory experiment (June 26th–July 24th) differed from the former only in its greater duration (4 weeks).

Table III.

Rat	Weight (g.) Before	Weight (g.) After		Uterus mg.		Vagina mg.		Preputial glands mg.		Substance
4006	44	88	92	58	62	98	85	33	33	Equilin
4007	52	115		84		110		44		
4008	38	68		58		74		27		
4009	46	100		60		87		27		
4010	36	90		51		58		32		
4011	41	85	93	47	47	79	85	26	35	Oestrone
4012	42	85		44		86		39		
4013	Died	—		—		—		—		
4014	39	105		51		89		41		
4015	Died	—		—		—		—		
4016	43	75	83	24	28	77	68	22	24	Oil
4017	47	98		42		85		16 (1 !)		
4018	37	68		19		52		15		
4019	44	82		36		68		26		
4020	37	90		17		59		26		

Two animals died in the oestrone group. With the exception of the vagina and to a less extent of the preputial glands, the increase of organ-weight was less than appeared in the former experiment; obviously because, the dose remaining constant throughout both experiments, the tissue hormone concentration became further reduced in the second experiment *pari passu* with greater increase of total body weight. The action of equilin on the uterus is again superior, and this time the preputial glands have also reacted.

The conclusion is that as a stimulus to the growth of secondary sex organs in the female rat equilin is certainly no less powerful than oestrone.

(*c*) *Opening of the vagina.*

Immature rats were used at about 3 weeks old. Five injections of 0·1 ml. were given (each containing either 2γ oestrone or 3γ equilin), two on June 5th, two on the 6th, one on the 7th. The weight was approximately the same in all cases (25 g.); observation continued until June 15th.

Table IV.

Rat	Oestrus	Vagina opening	Substance	Rat	Oestrus	Vagina opening	Substance
3879	Dioestrus	9. vi	Equilin	3885	Dioestrus	11. vi	Oestrone
3880	,,	9. vi		3886	,,	10. vi	
3881	,,	8. vi		3887	?	—	
3882	,,	9. vi		3888	Dioestrus	11. vi	
3883	,,	9. vi		3889	?	—	
3884	?	—		3890	?	—	

Clearly, equilin provoked opening of the vagina more regularly and consistently earlier than oestrone. Subsequent vaginal smears were in all cases negative, in agreement with Girard's observation [1933] that equilin and oestrone in equal doses by weight produce approximately the same effect.

(*d*) *Antimasculine effect.*

(1) *Experiments on young rats.* Three groups of five rats were used, 22–23 days old, comprising as many litter-mates as possible. One group was a control, the other two received respectively 0·5γ oestrone and 0·75γ equilin daily for 4 weeks in 0·1 ml. oil solution.

The antimasculine action is partially counteracted by the "paradoxical" effect (see section (*e*)), though the latter is weak at such low dosage. The average weight of the seminal vesicles decreased in the oestrone group, though not in the equilin group owing to the exceptional reaction of rat 3826.

The growth of preputial glands was stimulated in the oestrone group ("paradoxical" effect) and unaffected in the equilin group (*cf.* the female preputial glands in section (*b*)). A distinct "antimasculine" action on the testis, epididymis and remaining genitalia was seen in both groups but was more pronounced in the equilin-treated.

Table V.

	Weight (g.)			Testicle		Epididymis		Seminal vesicles		Preputial glands		Rest		
Rat	29. v	26. vi		mg.		mg.		mg.		mg.		mg.		Substance
3822	32	84		94		28		15		27		216		
3823	30	90		112		20		9		33		201		
3824	42	95	88	96	100	33	30	13	18	32	27	206	205	Equilin
3825	31	100		80		33		14		12 (1)		214		
3826	30	72		118		36		37!		16		189		
3827	31	85		218		76		14		66		331		
3828	Died	—		—		—		—		—		—		
3829	42	85	90	168	170	39	47	7	10	39	41	233	254	Oestrone
3830	35	95		131		32		10		22		208		
3831	36	95		161		40		8		36		245		
3832	26	80		641		120		28		41		474		
3833	32	90		890		115		19		30		360		
3834	40	95	91	760	780	98	115	14	17	33	32	420	409	Oil
3835	35	105		460		62		9		34		300		
3836	30	85		1160		179		14		22		490		

(2) *Experiments in adult rats.* These are uncomplicated by the "paradoxical" effect and require no longer than 2 weeks. In young rats we determine the retardation of the spontaneous genital growth with reference to control animals; this requires time. In adult rats an artificial atrophy is provoked.

Table VI.

	Weight (g.)			Testicle		Epididymis		Seminal vesicles		Preputial glands		Rest		
Rat	1. vi	15. vi		g.		mg.		mg.		mg.		g.		Substance
3858	240	215		2·10		460		127		98		1·89		
3859	210	190		1·58		370		87		39 (1)		1·36		
3860	185	170		1·53	1·71	320	380	61	94	57	84	1·20	1·45	Equilin
3861	237	220		2·17		460		97		99		1·50		
3862	240	205		1·10		290		96		35 (1)		1·31		
3863	214	200		1·80		480		126		108		1·51		
3864	210	200		2·12		470		116		78		1·94		
3865	238	235		2·30	2·01	660	530	138	110	96	90	2·18	1·78	Oestrone
3866	240	240		2·33		700		100		90		1·82		
3867	180	170		1·48		350		69		77		1·45		
3868	240	270		2·89		970		890		94		3·45		
3869	225	240		2·60		980		650		174		2·85		
3870	237	250		2·46	2·65	980	1000	640	720	198	122	3·12	3·16	Oil
3871	200	245		2·67		1090		690		121		3·23		
3872	Died	—		—		—		—		—		—		

Three groups of five rats were used (from unknown litters) receiving respectively 3·75γ equilin, 2·5γ oestrone and control oil.

"Antimasculine" action was clearly evident in all organs weighed and was more pronounced in the equilin group. The "antimasculine" activity of equilin, therefore, is at least as great as that of oestrone.

(e) *Paradoxical effect.*

This is a term which we have applied to the capacity of oestrone and allied substances to stimulate growth in certain parts of the genital tract of the male, especially the castrated male. We have already shown elsewhere [Freud *et al.*, 1933] that, apart from the preputial glands and their stratified epithelium, the reaction principally concerns the organs containing smooth muscle.

We used three groups of young recently castrated rats, some 26 days old (litter A) and some 24 days (litter B). The dose was 5γ oestrone and 7·5γ equilin twice daily in the respective groups. Duration of the experiment, 3 weeks.

Table VII.

Rat	Weight (g.) 30. v	Weight (g.) 22. vi	Litter	Seminal vesicles mg.	Prostate mg.	Preputial glands mg.	Substance
3837	27	60	A	54	6	31	
3838	32	75		37	5	12	
3839	30	55	B	72 ⎬ 53	6 ⎬ 6·2	21 ⎬ 23	Equilin
3840	31	79		54	6	35	
3841	36	80		50	7	15	
3842	32	85	A	52	5	34	
3843	34	75		60	7	32	
3844	25	60	B	62 ⎬ 57	5 ⎬ 6·6	17 ⎬ 32	Oestrone
3845	34	73		51	6	31	
3846	31	75		59	6	45	
3847	32	85	A	7	5	20	
3848	Died	—		—	—	—	
3849	33	81	B	5 ⎬ 5	3 ⎬ 4·25	39 ⎬ 24	Oil
3850	35	80		5	5	24	
3851	28	59		4	4	14	

By "prostate" is meant only the easily dissected ventral part of the gland; this grew very little, as expected, whereas the seminal vesicles grew very considerably in both treated groups. The preputial glands were augmented only by oestrone, as in some of the other experiments. The growth of the preputial glands, particularly in the male, is apparently stimulated by equilin less than by oestrone in oestrogenically equivalent unitage.

(f) *Growth of nipples.*

Stimulation of nipple-growth by oestrone and allied substances is most readily seen and measured in the (male) guinea-pig. The present experimental dose (1·5γ equilin, or 1·0γ oestrone, twice daily for 3 weeks), based on our former experience, was expected to be just sufficient to cause a distinct reaction, thereby demonstrating fine differences in activity. Table VIII states in millimetres the actual measurements obtained and shows almost mathematically identical activities for the two substances. However, two of the three animals in the equilin group secreted "milk" from the 4th or 5th to the 8th day after the last injections (which is remarkably late), whereas the oestrone group, as would be expected with so low a dosage, showed no secretion.

Equilin, therefore, stimulates the mammary gland at least as strongly as oestrone. The delayed secretion invites speculation concerning possible tissue storage of the hormone, on which point experience is lacking.

Table VIII.

Guinea-pig	Substance	Weight 31. v	Measurement of nipples (mm.) 31. v			Measurement of nipples (mm.) 7. vi		
3852	Equilin	662	2·3–3·4 and	2·0–2·9	2·2–3·0	2·9–4·2 and	3·0–4·4	2·8–4·0
3853		540	2·1–2·9	1·9–2·5		2·3–4·5	2·4–3·5	
3854		786	2·5–3·0	2·6–3·3		3·0–3·7	3·1–3·6	
3855	Oestrone	608	2·4–2·8	2·4–3·2	2·3–3·1	2·8–3·6	2·6–3·7	2·6–3·9
3856		536	2·3–3·2	1·9–2·8		2·4–4·0	2·8–4·0	
3857		784	2·8–3·1	2·5–3·4		2·5–3·8	2·8–4·2	
			14·6			21·6		
3852	Equilin	662	3·7–5·0 and	3·0–5·0	3·7–4·8	3·6–5·0 and	3·7–5·0	3·9–5·0
3853		540	3·7–5·1	3·2–4·6		3·6–5·1	3·0–4·8	
3854		786	4·2–4·2	4·2–5·0		4·8–4·8	4·8–5·2	
3855	Oestrone	608	4·2–4·6	3·7–4·5	3·65–4·3	5·0–5·5	4·6–5·0	3·9–4·9
3856		536	3·7–4·5	3·5–4·5		3·7–5·6	3·2–5·0	
3857		784	3·6–3·8	3·2–4·1		3·5–4·2	3·3–4·6	

(*g*) *Epithelial metaplasia of the male genital tract.*

This was noted after use of oestrone almost simultaneously by Lacassagne [1933, 1, 2], de Jongh [1933, 1934] and Burrows and Kennaway [1934]. Five prepubertally castrated adult male mice received 0·75γ equilin subcutaneously twice daily for 3 weeks, while four more received 0·5γ oestrone twice daily. The genitalia were examined by serial section with the following results.

(1) Stratified epithelium appeared in certain parts of the prostate (Lacassagne, de Jongh) of all mice, somewhat more conspicuously in the equilin group.

(2) The excretory ducts of the seminal vesicles showed similar changes (de Jongh) to a similar extent in both groups.

(3) Connective tissue changes in the ampulla of the vas deferens [Heringa and de Jongh, 1934] were of the same order in all treated animals.

(4) "Paradoxical" thickening of the vesicular smooth muscle (see section (*e*)) was somewhat weaker, though still distinct, in the equilin group. Similar experiments using rats produced the same order of change in both groups treated. The seminal vesicles were entirely used for serial section and therefore not weighed.

DISCUSSION.

The comparative biological activities of equilin and oestrone have been examined under varying experimental conditions and on various test objects, the minimum dose causing oestrus in mice being adopted as a basis of comparison in preference to a weight basis, for practical reasons.

Equilin is as active as or more active than oestrone in nearly every instance. Two exceptions are the growth of preputial glands (especially in the male) and, in less degree, the "paradoxical" growth of seminal vesicles in the mouse. Neither of these being material to the human subject, our results do not speak against the clinical use of equilin.

The possibility of course remains that human sensitivity may differ from mouse sensitivity in an opposite direction from that of the rat; in which case oestrone would naturally be preferable. The regrettable paucity of quantitative data concerning the action of oestrone in human subjects renders actual comparison impossible. As matters stand, equilin might even prove a more suitable therapeutic agent than oestrone. On the pharmacological evidence, equilin cannot be regarded as a harmful or even undesirable impurity in commercial oestrogenic preparations, which are standardised in mouse units (though were the comparative basis to be the minimum oestrogenic rat unit, equilin might be expected to show weaker antimasculine and uterine growth-promoting activity).

SUMMARY.

1. Equilin, compared with oestrone in equivalent dosage (calculated from their oestrogenic activities in mice), was more active in producing oestrus in the castrated rat, uterine and to some extent vaginal growth, vaginal opening in the young female rat, inhibition of the testes and male secondary sex organs of the male rat (with the few exceptions mentioned below), stimulation of the mammary gland of the guinea-pig and metaplasia of prostatic epithelium in the mouse.

2. The activities of equilin and oestrone were approximately equal as regards inhibition of preputial glands and seminal vesicles in the entire rat, "paradoxical" stimulation of the growth of seminal vesicles in the young castrated rat or of nipples in the guinea-pig, metaplasia of the vesicular duct epithelium of the mouse and changes in the connective tissue of the vas deferens of the mouse.

3. Oestrone was a more effective stimulus to the growth of preputial glands in the young female and to a greater extent in the male rat; and to the growth of seminal vesicles in the castrated mouse.

4. Equilin, when present in commercial oestrogenic preparations, cannot be regarded as an undesirable contamination.

REFERENCES.

Burrows and Kennaway (1934). *Amer. J. Cancer*, **20**, 48.
Freud, de Jongh and Laqueur (1933). *Ned. Tijdschr. Gen.* **77**, 1109.
Girard (1932). *Compt. Rend. Acad. Sci.* **194**, 909, 1020.
—— (1933). *Bull. Soc. Chim. Biol.* **15**, 562.
Heringa and de Jongh (1934). *Z. Zellforsch.* **21**, 629.
de Jongh (1933). *Acta Brev. Neerl.* **3**, 112.
—— (1934). *Ned. Tijdschr. Gen.* **78**, 1208.
—— Laqueur and de Fremery (1932). *Biochem. Z.* **250**, 448.
Lacassagne (1933, 1). *Compt. Rend. Soc. Biol.* **113**, 590.
—— (1933, 2). *Compt. Rend. Soc. Biol.* **114**, 870.
Laqueur (1935). (In the press.)

XLV. THE OXYTOCIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

V. RECOGNITION AS AN OXIDATION-REDUCTION SYSTEM.

By JOHN MASSON GULLAND
and SYDNEY STEWART RANDALL[1].

From the Biochemical Department, The Lister Institute, London.

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In continuation of the study of the effects of chemical reagents on the physiological activity of the oxytocic hormone, it was important to examine its behaviour with reducing agents. Amongst such reagents electrolytic hydrogen and hydrogen sulphide suggested themselves as being peculiarly suitable for trial on account of the mildness of the conditions under which they can operate. This communication describes the partial inactivation of the hormone by electrolytic hydrogen and by hydrogen sulphide; the changes which the hormone then undergoes when freed from the reagents or when treated with oxidising agents such as molecular oxygen or the oxidised forms of certain dyes; and finally the examination of the equilibria developed in mixtures of the hormone and indicators of known oxidation-reduction potentials.

Description of results.

The physiological activity of the hormone was diminished to about 55 % of its initial value by electrolytic reduction for 4 hours. No immediate change in activity occurred when the reduced solution was preserved from contact with air for some hours, but when oxygen was passed into the solution, the activity first rose to about 85 % and then fell to some 60 % of the initial value (Fig. 1).

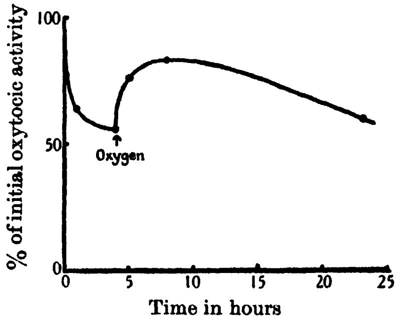

Fig. 1.

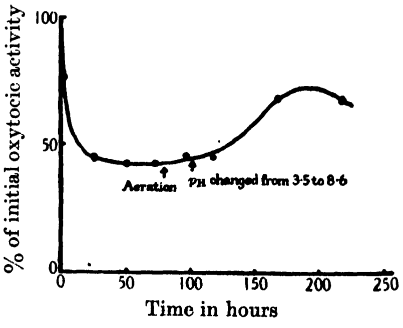

Fig. 2.

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[1] Lister Institute Research Student in Biochemistry.

Hydrogen sulphide, when added to a solution of the hormone at p_{H} 3·5, caused the physiological activity to fall at a moderate rate to 42 % of its initial value. At this point the activity then remained constant (Fig. 2 and Table I). Aeration of such solutions with currents of moist air to remove excess of hydrogen sulphide was followed in acid and in alkaline solutions by a slow temporary recovery of oxytocic activity, which probably rose to its maximum (about 75 % of the initial value) in the course of 100 hours. The activity then slowly decreased again, and the experiments were stopped. It might have been expected that the progressive gain in activity would have complicated the assays, but in practice no difficulty was experienced, since the rate of reactivation was so slow.

A different picture was presented by the results of the next series of experiments. Hitherto, no precautions had been taken to exclude air from the reduction, and all the solutions had been prepared from distilled water containing dissolved air. The reductions with hydrogen sulphide were therefore repeated using solutions which had been boiled to expel air. This modification did not greatly affect the value to which the oxytocic activity fell as the result of the reduction (about 45 %), but when samples were diluted with distilled water containing air, the physiological values rose so rapidly that it was quite impossible to assay the solutions by the customary method of arranging the contractions of the guinea-pig uterus in groups of four [Gulland and Newton, 1932]. A method of assay, elaborated in the experimental section, demonstrated clearly (Fig. 3) that the activity rose during 1 hour from about 45 % to about 80–90 %, that it then fell to about 55 % during the course of a further hour, and that it then remained apparently constant.

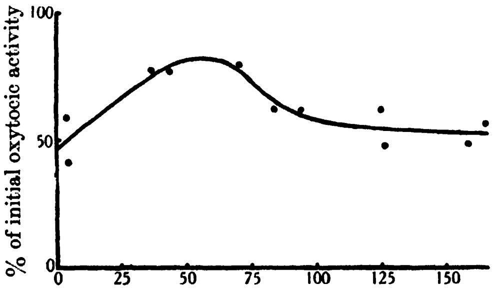

Fig. 3.

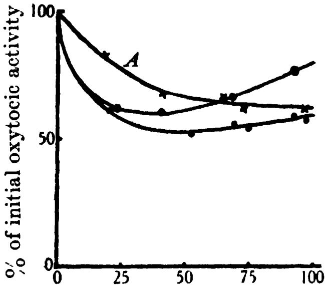

Fig. 4.

Both in this and the preceding series of experiments, the dilution of the reduced hormone solutions with ordinary distilled water resulted in a temporary gain in activity followed by a decrease. In the first series (reduction in presence of air) the gain and fall were slow, occupying together about 200 hours. In the second series, the gain and fall were rapid, occupying together about 2 hours. These divergences must be attributed to the presence or absence of air during the reduction, but no information is at present available as to the identity, or otherwise, of the changes responsible for the increases and subsequent decreases of activity in these experiments.

In view of the complicated results described above which might have been due to the effects of oxygen on the products of the reduction by hydrogen sulphide, it was necessary to study the reduction and subsequent behaviour of the hormone under conditions which remained anaerobic until the doses were introduced into the testing-bath for assay. Such conditions were realised by the use of boiled solutions cooled in nitrogen and by carrying out the reductions and dilutions in atmospheres of pure nitrogen. Under these circumstances and when the assay was performed immediately, the oxytocic value of a partially purified hormone solution decreased to about 55–60 % of its value after the reduction had proceeded for 40 hours and thereafter began slowly to increase again (Fig. 4). In exactly similar experiments with a less highly purified hormone solution containing more proteinoid material, the reduction proceeded less rapidly; the experiment was not prolonged sufficiently to ascertain the final value or to determine whether the activity again increased (Fig. 4, curve *A*). Evidently the course of the reduction was influenced by the presence of other substances.

Experiments were then made to ascertain whether the oxytocic activity was affected by the mere removal under anaerobic conditions of hydrogen sulphide from the reduced hormone solutions (55–60 %, above) and its replacement by pure nitrogen. Assays were made of diluted samples of reduced solutions immediately after the dilution. The solutions were then freed from hydrogen sulphide by repeated evacuations and fillings with nitrogen, and second assays made at intervals varying between 1 and 2½ hours from the first assays gave values for the activities which were different from those of the first assays. At first sight, it appeared as if hydrogen sulphide were affecting the response of the uterus to doses of the hormone, but this explanation was ruled out by demonstration that comparable and even much greater doses of hydrogen sulphide, introduced directly into the bath, did not modify the response to standard doses of hormone solution.

A study was therefore made of the changes in oxytocic value which occurred with passage of time when reduced hormone solutions were freed from hydrogen sulphide and kept under anaerobic conditions (Figs. 5 *a* and 5 *b*). The activity first increased, and the extent and rate of this reactivation in the case of a hormone solution which contained relatively much physiologically inactive material (Fig. 5 *a*) were greater than in the case of a purer solution (Fig. 5 *b*), indicating that reactivation was possibly due, at any rate in part, to interaction of the hormone with other substances present in the solution. The value to which the activity rose in the reactivations was less than 100 %, and this change was followed by a loss of activity, of which the final result was a value of about 50 %. In the case of the less pure hormone solution (Fig. 5 *a*), however, the

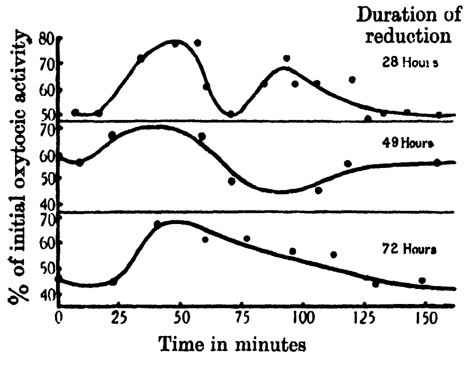

Fig. 5 *a*. Hormone solution 2.

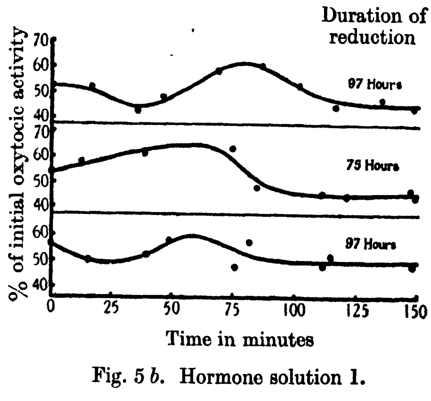

Fig. 5 *b*. Hormone solution 1.

exact progress of the changes of activity after the peak of reactivation is not clear. The curves of Fig. 5 *a* have been drawn as closely as possible to the experimental points, and it may be seen that when the duration of reduction was short there is evidence of a second peak, which became smaller and finally disappeared as the duration of the reduction with hydrogen sulphide was increased. It has not been possible to carry out sufficient experiments to establish this fact, and it is not necessary to investigate it further at present. If this peculiar behaviour is correct and is a property of the hormone, it must be investigated later with a purer specimen; if it is due to the action of other substances present in the solution, its full investigation is not a matter of immediate importance for this communication.

The experiments described above indicate that oxidation or reduction of at least one group in the hormone molecule was accompanied by, and presumably caused, variations in the oxytocic activity. A preliminary investigation of the oxidation-reduction potential of this system was made by studying the action of oxidised dyes on the hormone reduced by hydrogen sulphide. Methylene blue, for example, completely restored the activity of the reduced hormone, presumably by oxidation of the reduced group. Neutral red, on the other hand, effected only partial reactivation at p_H 3·6 and seemed to have little action at p_H 6·6 and 8·5. The examination of the redox system by this method was unreliable, however, partly because hydrogen sulphide itself is unsatisfactory as an indicator of redox potential, and partly because it might attack the molecule otherwise than by straightforward reduction. Moreover, neutral red is unsuitable as an indicator since its reduced form is unstable [Clark and Perkins, 1932]. Reliable results, however, were to be expected from assays of the oxytocic activity at equilibrium of mixtures of hormone solutions and the oxidised and leuco-forms of reversible oxidation-reduction indicators. Fig. 6 shows how the oxytocic activity of the hormone varies with changes in the electrode potential of the medium between +0·04 and −0·26 v. at p_H 6. The left-hand portion of the curve represents the theoretical relationship between the percentage of the oxidised form of a redox system and the potential in volts, drawn in this case to a scale such that the values representing the oxidised and reduced forms correspond to those denoting the highest and lowest oxytocic activities determined. The right-hand section of the curve is the mirror image of the left-hand portion. Equilibrium was rapidly attained, except at potentials of −0·12 and −0·16 v., when an upward drift towards increased activity oc-

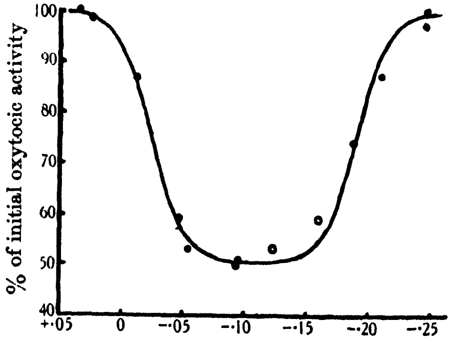

Fig. 6.

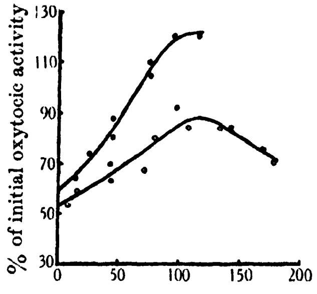

Fig. 7.

curred so rapidly that assay by the normal procedure was impossible. These drifts (Fig. 7) were followed by the method of assay developed in the reactivations after reduction by hydrogen sulphide, and the values denoted by open circles in Fig. 6 were estimated by extrapolating these curves to zero time.

At potentials of $+0{\cdot}35$ (phenolindophenol) and $-0{\cdot}45$ v. (methyl viologen) (not shown in Fig. 6) the oxytocic activity was 100 %.

Discussion.

The experiments described above demonstrate that the oxytocic hormone contains one oxidation-reduction system, having $E_0'-0{\cdot}025$ v. at p_H 6. When the system is oxidised the oxytocic activity is 100 %, when reduced, 50 %. It is interesting to recall that Johnson *et al.* [1927] concluded that oxidation-reduction processes constitute an important factor in the regulation of the tone of uterine muscle.

The results might also be interpreted as showing that the hormone molecule contains a second oxidation-reduction system having $E_0'-0{\cdot}190$ v. at p_H 6. On this view, the oxytocic activity would be 100 % when both systems are oxidised or reduced, and 50 % when one is oxidised and the other reduced. The existence of the second system is possible, but there are certain objections to its unqualified acceptance, and these are discussed below.

The oxytocic activity remains at 100 % after treatment of the hormone with methyl viologen at a potential of $-0{\cdot}45$ v. or with alkaline glucose. On the other hand, electrolytic reduction for 4 hours lowers the activity to 56 %. These facts are not in harmony if the reagents only attack the oxidation-reduction systems. If the hormone contains two redox systems, as seems to be indicated by the experiments with redox potential indicators and alkaline glucose, electrolytic reduction should not have decreased the activity. Possible explanations of the apparent anomaly are that the duration of the electrolytic reduction was insufficient to allow both systems to be reduced or that its effect was complicated by exposure of the reduced hormone to air at the surface of the liquid in the reduction vessel. In the hope of settling this point, the hormone was reduced catalytically with hydrogen and platinum black for many hours; the activity fell rapidly, and remained at 50 %. This fact seemed at first to support the results of the electrolytic reduction and to show that the hormone contained only one redox system, the activity being 50 % when this was reduced. In that case, the increasing oxytocic activities corresponding to increasingly negative potentials, as shown by the right-hand portion of Fig. 6, might be the result of oxidation of the reduced system of the hormone through a rapid transfer of oxygen by the more negative redox potential indicators when the dose is introduced into the oxygenated Ringer solution in the testing bath.

It was demonstrated in the following ways that transfer of oxygen is not alone responsible for the higher activities at the more negative potentials. The hormone was reduced to an activity value of 50 % by indigodisulphonate at a potential of $-0{\cdot}09$ v. (Fig. 6) and was then mixed anaerobically with an air-free solution of oxidised methyl viologen. No marked change in the potential of the solution occurred, as would have been shown by a large increase in the blue colour of the solution, but the oxytocic activity rose to 74 %. In an exactly similar experiment in which rosinduline replaced methyl viologen the activity rose to 59 %. These results indicate that transfer of oxygen to the hormone by the dye may occur to some extent, and the mutual relationship of the values in the two experiments supports this view. It was expected, however, that complete reactivation of the hormone would have occurred if transfer of oxygen were the sole cause of the increasing oxytocic activity with the more negative indicators.

Further attempts were made to investigate the question of oxygen transfer by making assays under conditions in which the supply of available oxygen was considerably reduced. Johnson *et al.* [1927] record that when the isolated guinea-pig uterus is suspended in Ringer solution through which nitrogen, instead of oxygen, is passing, it contracts and remains in that state of tonus until oxygen is admitted. Attempts to repeat these observations were unsuccessful, even when nitrogen, freed from oxygen as described in this communication, was used and when the Ringer solution was covered with a layer of medicinal paraffin to exclude air. Spontaneous contractions of the uterine horn did occur, only to be followed by relaxation in the usual manner. We do not wish to stress this difference between our observations and those of Johnson *et al.*, or to offer any adverse criticism of their results. It has, however, been possible to make completely satisfactory assays of hormone solutions using a uterine horn suspended in Ringer solution covered with paraffin and saturated by a rapid stream of pure nitrogen. The only oxygen available must either (*a*) have been absorbed by the horn during its momentary exposure when the bath was emptied between doses or subsequently from the fresh Ringer solution, or (*b*) have remained dissolved in the Ringer solution in the bath at 37° for about 8 or 10 minutes in a rapid stream of nitrogen. This method was utilised to assay some hormone solutions which had been reduced with hydrogen sulphide or used in electrode potential experiments. No difference within the limits of experimental error could be detected between the activities of solutions assayed in this and in the normal way, except in the case of the indigodisulphonate-methyl viologen experiment described above; in this case, the activity was 61 %, instead of 74 % determined by the normal method.

It seems therefore that transfer of oxygen by the more negative indicators may cause oxidation of the hormone and consequent increase in oxytocic activity, but that this transfer is insufficient to account for the apparent activity of 100 % exhibited by the hormone at potentials of −0·247 v. and lower.

This conclusion strengthens the possibility that the hormone molecule contains two oxidation-reduction systems, and support for this view might be derived from the course of anaerobic reduction by hydrogen sulphide—initial decrease of activity to 50–60 %, followed by a slow increase. The chief evidence to the contrary was that catalytic or electrolytic reduction lowered the activity to about 50 %, instead of leaving it unchanged at 100 %. It was shown as follows that this was probably due to adsorption of the hormone or to its destructive reduction. A hormone solution which had been reduced by platinum black and hydrogen and exhibited 50 % of its initial activity was mixed anaerobically with oxidised methylene blue in order to convert the redox system completely into its oxidised form as in the experiments after reduction with hydrogen sulphide. The activity first fell, a value of about 33 % being recorded, and then rose again to 50 %, where it remained constant. These changes would have been predicted on the assumption that two redox systems in half the hormone molecules present in the solution had been fully reduced, resulting in an activity of 100 % as regards those molecules, and that the other half of the hormone molecules had been adsorbed on the catalyst or destroyed. In agreement with this view, it was found that oxidised rosinduline, added anaerobically to the reduced hormone solution (activity 50 %), did not alter the activity. Further, an attempt to restore full activity to an electrolytically reduced solution (value 70 %) by adding oxidised methylene blue only raised the activity to 83 %, indicating that some hormone molecules had been destroyed. These facts are in agreement with the existence of two redox systems. On the other hand, the addition of reduced rosinduline to the catalytically reduced hormone solution lowered the activity from 50 % to about 20 %, a fact which is not in harmony with these views.

Our present opinion is that the hormone contains one oxidation-reduction system, that it may also contain a second group which readily undergoes reduction, and that the states of oxidation of these groups are intimately connected with the physiological activity of the hormone. In addition, transfer of oxygen to the reduced hormone may occur in the testing bath through the agency of the more negative redox potential indicators.

The decision as to whether the drift of the oxytocic activity of the hormone at electrode potentials between about $-0 \cdot 11$ and $-0 \cdot 18$ v. or after reduction by hydrogen sulphide is an inherent property of the molecule or results from the presence of other substances must be postponed until purer hormone solutions are available. The subsequent inactivations which follow these increases in activity are also intimately linked with this question. Whatever the explanation may be, it is clear that molecular oxygen is not a necessary factor, since both these changes can occur in its absence. On the other hand, oxygen may well take part in the aerobic reactivations, and undoubtedly it is responsible for the existence of the hormone in its oxidised state in posterior pituitary extracts. It would be unwise to attempt an explanation of these activations and inactivations without a knowledge of the simultaneous electrode potentials of the solutions in which they were occurring. Possibly measurements of these may throw light on these questions.

EXPERIMENTAL DETAILS.

Hormone solutions.

Three solutions of the hormone were used in this investigation. One, called hormone solution 1, was prepared according to Kamm *et al.* [1928], and corresponded in its state of purification to that used by Gulland and Newton [1932]. It contained 300 oxytocic units per ml., associated with 5·65 mg. of solid when dried to constant weight.

The second solution, called hormone solution 2, was prepared by extracting dried, powdered posterior lobe (1·5 units per mg.) with 0·25 % acetic acid, concentrating the extract and precipitating by saturation with ammonium sulphate as described by Kamm *et al.* [1928]. The precipitate was then thoroughly extracted with 98 % acetic acid, and the extract was mixed with ether (2·5 vols.) and light petroleum (5 vols.). The solid thus precipitated was washed with a mixture of ether and light petroleum and was dissolved in a convenient volume of water at p_H 3. This solution therefore contained more inactive material than hormone solution 1, the actual content being 28·3 mg. of solid when dried to constant weight associated with 450 units per ml. The amount of ammonium sulphate was 3·9 mg. per ml., based on estimations of sulphate ion as barium sulphate, so that the weight of glandular material was 24·4 mg. per ml.

The third solution, hormone solution 3, prepared as described by Kamm *et al.*, contained 900 units per ml., associated with 3·67 mg. of solid.

Electrolytic reduction and reactivation by oxygen (Fig. 1).

The apparatus used was a rectangular three-compartment cell of the type used for electrodialysis. The electrodes were platinum foil, each measuring

2·5 × 2·5 cm. and the compartments were cooled by glass coils containing flowing water. The dividing membranes consisted of cellophane No. 400, and each compartment contained 10 ml. of liquid. Experiments on the electrodialysis of the hormone will be described at an early date in another communication.

The following description is typical of several experiments. The cathode chamber contained a mixture of hormone solution 2 (0·2 ml. containing 90 units) and $M/10$ sodium acetate solution (0·1 ml.) diluted to 10 ml. The centre compartment was filled with water (9 ml.) and $M/10$ acetate buffer at p_H 3·5 (1·0 ml.), and the anode chamber contained $N/1000$ acetic acid (10 ml.). The current was maintained at 50 milliamps, the voltage across the cell being 60 v. in the early part of the experiment and somewhat higher after the first half-hour. The temperature remained at 15°. The cathode solution was kept adjusted to p_H 9 by passing in carbon dioxide as required.

After the reduction had proceeded for 1 hour, the cathode solution was transferred quantitatively to a 25 ml. flask, brought to p_H 3·5 by the addition of 0·5 ml. of $N/10$ hydrochloric acid and diluted to 25 ml. When diluted and assayed, it had 66 % of the total activity, and this value remained unchanged when the undiluted solution, which still contained dissolved hydrogen, was kept in a cooled vessel for 18 hours.

When the reduction was prolonged for 4 hours and the cathode solution was treated as before, it contained only 56 % of the total activity. In both cases the liquid in the anode compartment exhibited no activity, and the centre compartment contained less than 1 % of the total number of units.

A stream of moist oxygen was passed for 1 hour through the cathode solution which had been reduced for 4 hours. The assay of a sample, taken at the end of this period and tested at once, showed that the activity had risen to 76 %, but after this oxygenated solution had been kept at 0° for 18 hours, the activity had fallen to 60 %. In a parallel experiment, the activity after oxygenation for 1 hour was 75 % and 3 hours later 83 %.

Aerobic reduction by hydrogen sulphide and reactivation by aeration (Fig. 2).

Mixtures of hormone solution 1 (0·1 ml. containing 30 units), $M/10$ acetate buffer at p_H 3·5 (0·5 ml.) and hydrogen sulphide solution (1·0 ml. saturated at 20°) were diluted to 2·5 ml. and kept at room temperature. The results are summarised in Table I.

Table I.

EXPERIMENT 1.			EXPERIMENT 2.		
Time hours	Treatment	Activity %	Time hours	Treatment	Activity %
2·5	Sample taken	76	2	Sample taken	75
26·5	„	46	7	„	50
50·5	„	42	25	„	42
75	„	43	25·5	Aeration by moist air for 1 hour at p_H 3·5	—
80	Aeration by moist air for 45 mins.	—	26·5	Sample for $Ce(OH)_2$ exp.	—
98	Sample taken	45	50·5	Sample taken	50
102	p_H adjusted to 8·2	—	75	„	53
119	Sample taken	45			
170	„	68			
218	„	67			

It is evident that the reactivation occurs very slowly in acid or alkaline solution. In order to ascertain whether the rate of reactivation would increase and the activity be restored completely if the formation of hydrogen peroxide were avoided or its amount, if formed, were diminished, a portion (1·0 ml.) of the solution from Exp. 2, which had been reduced for 25 hours and then aerated was adjusted to p_H 8·2 with $N/10$ sodium hydroxide and then mixed with borate buffer at p_H 8·2 and a suspension of cerous hydroxide prepared from $M/50$ cerous sulphate (1·0 ml.). The suspension was shaken, and samples (not shown in Table I) were taken at 49 and 73 hours, centrifuged, diluted and assayed; the values for activity were 60 and 46 % respectively. The results of this experiment seemed to indicate that reactivation was more rapid in presence of cerous hydroxide. This point must not be stressed, however, since it is possible that the rate of oxidation was accelerated at the surface of the finely divided cerous hydroxide. In addition, no information could be obtained as to the adsorptive effect of cerous hydroxide on the reduced hormone or on the oxidised hormone formed in its presence.

Anaerobic reduction with hydrogen sulphide and reactivation.

The oxygen-free nitrogen used in all the anaerobic experiments was prepared by storing nitrogen (from a cylinder) over alkaline pyrogallol, scrubbing it thoroughly with alkaline pyrogallol immediately before use, passing it through a roll of reduced copper gauze in an electric furnace at a low red heat, and then washing it with air-free water. When purified in this way, the nitrogen gave no coloration with alkaline pyrogallol.

All solutions used in the anaerobic experiments were rendered free from oxygen as follows: they were either boiled and cooled quickly while a rapid stream of nitrogen passed through them, or if that procedure were unsuitable, they were repeatedly evacuated and saturated with nitrogen.

Reduction. The hormone solution (0·1 ml.) was mixed with $M/10$ acetate buffer at p_H 3·5 (0·5 ml.) and water (0·9 ml.). Part (0·3 ml.) of this solution was then freed from air and mixed with a saturated solution (0·2 ml.) of hydrogen sulphide in air-free water in a small tube swept out by nitrogen. The tube was then tightly stoppered and placed in a thermostat at 20°. A number of tubes prepared in this way at the same time formed the material for one experiment. After the reduction had proceeded for the desired time, one tube was introduced into a separating funnel containing air-free water (24·5 ml.) in an atmosphere of nitrogen. By evacuating the funnel, the stopper of the small reduction tube was released, care being taken not to allow hydrogen sulphide to escape from the funnel; the contents of the tube and funnel were thoroughly mixed, and the funnel was again filled completely with nitrogen. These experiments were repeated many times using hormone solutions 1 and 2, and some of the results, which differed slightly, are recorded in Fig. 4. These assays were not complicated by changes due to reactivation, presumably because these, if they occurred at all, were slow in presence of hydrogen sulphide.

Aerobic reactivation. In the early experiments on the reduction described above, the contents of the small tubes were diluted with air-containing water (24·5 ml.) and not as described above. Under these conditions it was observed that the activity seemed to have increased during the first hour after the dilution and then to have fallen at the end of 2 hours to approximately the original value of the reduced solution. The rate of change was too rapid to allow assays to be made by the customary procedure of arranging contractions of the uterus in groups of four [Gulland and Newton, 1932], but it was possible to obtain a moderately accurate estimate of the activity at any given time after dilution (*a*) by making assays simultaneously on two uterine horns previously brought to a satisfactorily sensitive condition by doses of standard hormone solution; and (*b*) by arranging doses of the solutions either in the order experimental,

experimental, standard, standard, or in the order experimental, standard, standard, experimental. From the charts thus obtained, the activity values of the experimental solutions were estimated by direct comparison of the heights of contractions evoked by them and by the adjacent doses of standard solution. It is evident that the results obtained by this procedure cannot be so reliable as those reached by the customary method of assay.

A number of experiments were made in this way, and in some cases the assays were made by two observers working simultaneously, but independently, with different uterine horns. These changes are illustrated by Fig. 3, from which it is clear that after reduction with hydrogen sulphide and dilution with aerated water the activity rose during 1 hour and then fell to a steady value close to that of the original reduced solution.

Anaerobic reactivation. A series of reductions of hormone solutions 1 and 2 was carried out as described above, and the reduction tubes were exploded as before in air-free water in an atmosphere of nitrogen. As soon as the contents of the separating-funnel had been mixed, a dose was withdrawn without exposing it to the air and inserted immediately into the testing-bath. After some practice it was possible to arrange that this dose should cause a contraction of which the height lay between two adjacent doses of standard solution. The activity of the reduced sample was thus ascertained with some measure of certainty, since in these experiments only those uterine horns were used which at the beginning of the assay were not only giving uniform responses to identical standard doses but which also exibited a satisfactory degree of sensitivity.

The anaerobic removal of doses of the experimental solutions from the tube below the tap of the separating funnel was easily accomplished by means of a 1 ml. hypodermic syringe graduated in 1/100 ml.

The remainder of the solution was at once freed from hydrogen sulphide by four successive evacuations and replacements by nitrogen, and the changes in activity were followed simultaneously in two uterine horns as described in the case of the aerobic reactivation experiments. Some results are given in Figs. 5 *a* and 5 *b*.

Action of dyes on the hormone after anaerobic reduction (*Table II*).

Hormone solution 2 (0·1 ml.), $M/10$ acetate buffer at p_{H} 3·5 (0·5 ml.), and hydrogen sulphide solution (1·0 ml., saturated at 20°) were mixed in a flask swept out by nitrogen. The mixture was diluted to 2·5 ml. with air-free water, and kept in a thermostat at 20°; the period of reduction varied between 24 and 48 hours in different experiments.

Methylene blue at p_H *4·2 and neutral red at* p_H *3·6.* The reduced hormone solution (1·0 ml.) was transferred without being exposed to air to a specially constructed tube swept out by nitrogen and containing air-free water (8 ml.). The tube was then evacuated and refilled with nitrogen four successive times, and whilst it was still under slightly reduced pressure an air-free, $M/50{,}000$ aqueous solution (1·0 ml.) of the oxidised form of the dye was added from a burette fixed in the stopper. Some minutes later (accurately timed), the p_{H} was determined, and a sample was removed anaerobically by a tap in the bottom of the tube and diluted for assay. Difficulties due to changes in activity were not experienced in making assays in the presence of these dyes, which seemed to stabilise the oxytocic value.

A control experiment was carried out simultaneously on another portion of the same reduced solution; the dye solution was replaced by water, and care was taken to ensure that the conditions as to time during which the dye acted and rates of evacuation and refilling were copied as faultlessly as possible. These precautions were necessary in view of the reactivation which occurs when the hydrogen sulphide is removed.

Neutral red at p_H *6·6 and 8·6.* These experiments were carried out similarly to those described above, except that the dye was added as a mixture of $N/10$ sodium hydroxide (0·1 ml.) and 2·0 ml. of $M/50{,}000$ neutral red in $M/5$ sodium phosphate buffer at p_H 6·6 or in Palitzsch's borate buffer [Clark, 1928] at p_H 8·6 respectively. Control experiments were made in the same way.

Table II.

Dye	p_H	Time of standing with dye mins.	% activity of control	% activity with dye
Methylene blue	4·2	10	69	104*
,,	4·2	5	50	100
Neutral red	3·6	4	55	71†
,,	3·6	5	64	78‡
,,	6·6	5	67	65
,,	6·6	5	63	69
,,	8·5	5	42	57
,,	8·5	4	70	69

* 100 % after remaining 1·5 hours in nitrogen.
† 88 % ,, 24 ,,
‡ 80 % ,, 21 ,,

Table III. *The relationship between oxidation-reduction potential and oxytocic activity.*

Dye	p_H	% oxidised form	E'_0	Correction K	E_h	% activity
Phenolindophenol	6·0	99	+0·29	+0·060	+0·35	96
Methylene blue[1]	6·0	25	+0·047	−0·014	+0·033	100
	6·0	14	+0·047	−0·024	+0·023	97
Indigotetrasulphonate[2]	6·0	20	+0·006	−0·018	−0·012	87
Indigotrisulphonate[2]	6·0	20	−0·028	−0·018	−0·046	59
	6·0	12·5	−0·028	−0·026	−0·054	53
Indigodisulphonate[2]	3·5	15	+0·080	−0·023	+0·057	55
	6·0	13·5	−0·069	−0·025	−0·094	51
	6·0	15	−0·069	−0·023	−0·092	50
Brilliant alizarin blue[3]	6·0	30	−0·112	−0·011	−0·123	53*
Anthraquinone-β-sulphonate[4]†	6·0	70‡	−0·170	+0·011	−0·159	59*
	3·5	20	−0·040	−0·018	−0·058	73
	5·6	20	−0·160	−0·018	−0·178	74
Rosinduline 2G[5]	6·0	70‡	−0·221	+0·011	−0·210	87
	6·0	14	−0·221	−0·025	−0·246	97
	6·0	12·5	−0·221	−0·026	−0·247	100
	6·0	20	−0·221	−0·018	−0·239	106§
Methyl viologen[6]	10·0	40 approx.	−0·446	−0·005	−0·45	100

[1] Clark *et al.* [1925].
[2] Sullivan *et al.* [1923].
[3] Michaelis [1930].
[4] Conant *et al.* [1922].
[5] Michaelis [1931].
[6] Michaelis and Hill [1933].

* During the assays of these experiments the activity drifted upwards, and these values were estimated by extrapolating to zero time the curves representing the drifts.

† Anthraquinone-β-sulphonate is practically colourless, and it was assumed that the oxidised form was present to an extent of 20 % of the total dye in the reaction mixture, since this was the average figure for a series of experiments made with coloured indicators at the same period.

‡ 0·5 ml. of the oxidised dye solution was mixed with the hormone solution, and 0·5 ml. of the leuco-dye solution was added from the burette.

§ This assay was made under as completely anaerobic conditions as possible. Nitrogen, free from oxygen, was substituted for the oxygen passing through the Ringer solution in the testing-bath, and a layer of medicinal paraffin covered the surface of this Ringer solution.

Oxidation-reduction potential of the hormone system (Table III).

These experiments were made in an apparatus in which the following series of operations could be carried out in an atmosphere of air-free nitrogen with solutions which had previously been freed from air. A $M/5000$ aqueous solution of the leuco-dye, prepared by reduction with platinised asbestos and hydrogen, was filtered through a sintered glass plate into a burette. 1·0 ml. was added to a mixture of the hormone solution (1·0 ml. of a 1/100 dilution of solution 2 or 0·5 ml. of a 1/100 dilution of solution 3, 4·5 units in each case), $M/10$ buffer solution (1·0 ml.) and water (7·0 or 7·5 ml.), the final volume being 10·0 ml. In every case oxidation of the leuco-dye occurred, and the percentage of oxidised form present in the reaction mixture was estimated colorimetrically. The total molarity of the dye in the mixture was 1/50,000. Temperature was 18° approximately. The samples for assay were removed at a tap in the reaction vessel without being exposed to air and were introduced immediately into the testing bath, the dilutions having been suitably arranged to enable this to be done. Equilibrium appeared to be attained immediately in most cases, but at potentials of $-0{\cdot}12$ and $-0{\cdot}16$ v. the activity value drifted rapidly (see footnote to Table III), and a slow drift was also observed at $-0{\cdot}19$ v.

The effects of adding oxidised potential indicators to the hormone reduced with indigodisulphonate.

A $M/5000$ aqueous solution (1·0 ml.) of leuco-indigodisulphonate was added to an air-free mixture of a dilution of hormone solution 3 (0·5 ml., 4·5 units) $M/10$ acetate buffer at p_H 6 (1·0 ml.) and water (6·5 ml.) in the apparatus used in the previous experiments. An air-free $M/5000$ aqueous solution (1·0 ml.) of methyl viologen was then added, and assays of oxytocic activity were made in the normal way and in a stream of nitrogen (see Discussion). The values were 74 and 61 % respectively.

Addition of a $M/5000$ aqueous solution (1·0 ml.) of oxidised rosinduline 2 G instead of methyl viologen gave a solution which assayed at 59 %.

Reduction with hydrogen and platinum black.

The catalyst was a strip of platinum foil (8 × 0·5 cm.) coated electrolytically with a thin film of platinum black and washed by electrolysis in dilute sulphuric acid and then distilled water. A mixture (20 ml.) of a dilution of hormone solution 3 (2·22 ml., 20 units), $M/10$ acetate buffer at p_H 6 (2·0 ml.) and water was shaken mechanically with the catalyst in an atmosphere of hydrogen. After 1¼ hours an assay showed the activity to be 59 %, and after a further period of reduction of 11 hours the activity was 50 %.

Equal volumes of this reduced hormone solution and $M/5000$ solutions of potential indicators were mixed anaerobically and the results are summarised in Table IV.

Table IV.

Potential indicator	% of initial activity	Time elapsed from mixing indicator with reduced hormone mins.
Oxidised rosinduline	50	3
	50	30
Oxidised methylene blue	33	6
	50	60
Reduced rosinduline	20	6
	20	60

Electrolytic reduction and reactivation by methylene blue.

Hormone solution 2 was reduced electrolytically, as described previously, until an assay showed that the activity was 70 %. The reduced solution (5 ml.) was freed from dissolved hydrogen by repeated evacuation and saturation with nitrogen and was mixed anaerobically with a $M/5000$ aqueous solution (2·0 ml.) of methylene blue. An assay showed the activity to be 83 % of its initial value.

SUMMARY.

1. The oxytocic activity of the hormone falls to about half its initial value when the molecule is reduced by hydrogen sulphide. The reduced hormone is completely reactivated by oxidised methylene blue.

2. The hormone is also reduced by catalytic or electrolytic hydrogen, the activity again falling to about 50 %. Part of this decrease in activity is probably due to destruction of the hormone or to its adsorption by the catalyst or electrode.

3. The reduced hormone is partially reactivated when it is exposed to air or when the hydrogen sulphide is removed under anaerobic conditions, These reactivations are followed by inactivations. These processes vary according to the circumstances in which they occur and the conditions under which the hormone was reduced.

4. Assays of the oxytocic activity of the hormone in presence of indicators of known oxidation-reduction potential indicate that the hormone molecule contains a redox system having $E_0' \mp 0{\cdot}025$ v. at p_H 6. The activity is 100 % when this is oxidised and 50 % when it is reduced.

5. Assays of the oxytocic activity of the hormone at potentials between $-0{\cdot}10$ and $-0{\cdot}25$ v. show that the activity increases from 50 to 100 % over this range. This may indicate the presence in the molecule of a second group which readily undergoes reduction, but the interpretation of the results is complicated by the possibility that some oxidation of the reduced hormone in the testing bath may occur through a rapid transfer of oxygen to it by the more negative redox potential indicators.

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REFERENCES.

Clark (1928). The determination of hydrogen ions, 3rd ed. (Baillière, Tyndall and Cox.)
—— Cohen and Gibbs (1925). *U.S. Pub. Health Rep.* **40**, 1131.
—— and Perkins (1932). *J. Amer. Chem. Soc.* **54**, 1228:
Conant, Kahn, Fieser and Kurtz (1922). *J. Amer. Chem. Soc.* **44**, 1382.
Gulland and Newton (1932). *Biochem. J.* **26**, 337.
Johnson, McClosky and Voegtlin (1927). *Amer. J. Physiol.* **83**, 15.
Kamm, Aldrich, Grote, Rowe and Bugbee (1928). *J. Amer. Chem. Soc.* **50**, 573.
Michaelis (1930). *J. Biol. Chem.* **87**, 713.
—— (1931). *J. Biol. Chem.* **91**, 369.
—— and Hill (1933). *J. Gen. Physiol.* **16**, 859.
Sullivan, Clark and Cohen (1923). *U.S. Pub. Health Rep.* **38**, 1669.

XLVI. THE OXYTOCIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

VI. FURTHER STUDIES OF THE ACTION OF OXIDISING AND REDUCING AGENTS.

By JOHN MASSON GULLAND
AND SYDNEY STEWART RANDALL[1].

From the Biochemical Department, The Lister Institute, London.

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In Part V [Gulland and Randall, 1935] it was shown that the molecule of the oxytocic hormone certainly contains one, and may possibly contain two, oxidation-reduction systems, although the existence of the second is not definitely established.

The present communication describes the actions on the hormone of some oxidising and reducing agents, which were studied in the hope that they might throw light on the nature of the groups forming the oxidation-reduction system, or systems.

EXPERIMENTAL.

Sulphur dioxide and sodium sulphite (Fig. 1). Sodium sulphite at p_H 8·4 caused irreversible and complete inactivation of the hormone in 5 minutes. Sulphur dioxide at p_H 3·5, on the other hand, caused a slower inactivation to about 60 %, which was followed by reactivation and finally by inactivation to 3·6 % after 24 hours.

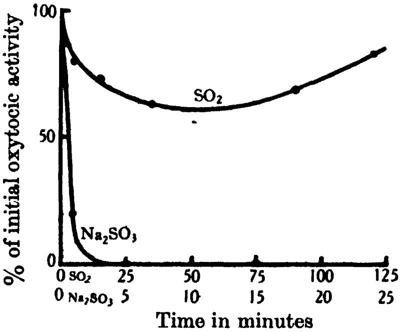

Fig. 1. Action of sulphur dioxide and sodium sulphite.

SO_2 at p_H 3·5 Na_2SO_3 at p_H 8·4

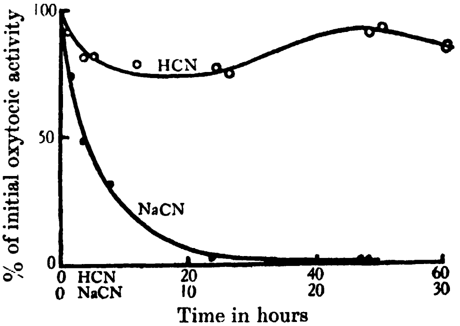

Fig. 2. Action of sodium cyanide and hydrogen cyanide.

HCN at p_H 3·5 (2 exps.) NaCN at p_H 7·8 (2 exps.)

At p_H *3·5.* The following solutions were placed in this order in a 2·5 ml. graduated flask: $M/10$ sodium acetate buffer at p_H 3·5 (0·5 ml.), $N/10$ hydrochloric acid (0·25 ml.), water (0·9 ml.), $N/10$ sodium sulphite (0·5 ml.), and a

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[1] Lister Institute Research Student in Biochemistry.

Kamm[1] solution of the hormone (0·1 ml.), containing 300 oxytocic units associated with 5·65 mg. of solid per ml. (called Kamm solution 1 throughout this paper). The mixture was diluted to 2·5 ml. with water, the final p_H being 3·5, and the stoppered flask was placed in a thermostat at 20°. Samples were diluted to a convenient volume for assay[2].

At p_H *8·4.* Palitzsch's sodium borate buffer [Clark, 1928] at p_H 8·4 (0·5 ml.), $N/10$ sodium sulphite (0·5 ml.), water (1·0 ml.) and Kamm solution 1 (0·1 ml.) were mixed, diluted to 2·5 ml. (p_H 8·4) and placed in a thermostat at 21°.

Sodium cyanide and hydrogen cyanide (Fig. 2). The hormone underwent a slow, irreversible and probably complete inactivation when treated with sodium cyanide at p_H 7·8. Hydrogen cyanide at p_H 3·5 slowly caused a temporary decrease in activity; this was followed by a slight reactivation and then by a fall to an oxytocic value of about 82 %, at which level the activity seemed to remain constant. In both series of experiments the samples were freed from hydrogen cyanide by aeration at p_H 3·5, a procedure adopted before the susceptibility of the reduced form of the hormone had been recognised. It is possible that some alteration of activity may have occurred during this aeration in the case of the experiments at p_H 3·5, though probably not in the case of those at p_H 7·8. Even if this were so the essential nature of the results at p_H 3·5 remains clear.

At p_H *3·5.* $M/10$ sodium acetate buffer at p_H 3·5 (0·5 ml.), $N/10$ hydrochloric acid (0·45 ml.), $N/10$ sodium cyanide (0·5 ml.) and Kamm solution 1 (0·1 ml.) were mixed, diluted to 2·5 ml. and placed in a thermostat at 20°. The p_H was 3·5. Samples were aerated with a current of moist air for 30–45 minutes until free from cyanide (Prussian blue test; sensitivity, 1 in 5×10^4) and were suitably diluted and assayed.

At p_H *7·8.* Palitzsch's sodium borate buffer at p_H 8·2 (0·5 ml.), $N/10$ hydrochloric acid (0·37 ml.), $N/10$ sodium cyanide (0·5 ml.) and Kamm solution 1 (0·1 ml.) were mixed, diluted to 2·5 ml. and kept in a thermostat at 20°. Samples were adjusted to p_H 3 by means of $N/10$ hydrochloric acid and were then aerated as before, diluted and assayed.

Hydrogen peroxide (Fig. 3). The general character of the interaction of the hormone with hydrogen peroxide was the same at p_H 3·5 and at p_H 8·2. A period of inactivation was followed by one of reactivation, after which the activity again declined. The only essential difference between the courses of these reactions was the increased rate at which the changes occurred in the alkaline medium. It should be noted that estimations of the concentrations of hydrogen peroxide made by the permanganate method [Treadwell and Hall, 1928] throughout the experiments showed that the concentrations remained unchanged within the limits of the experimental error of the method.

At p_H *3·5.* $M/10$ sodium acetate buffer at p_H 3·5 (0·5 ml.), $M/10$ hydrogen peroxide (0·2 ml.) and a Kamm hormone solution (0·1 ml.) containing 90 units associated with 0·37 mg. of solid were mixed and diluted to 2·5 ml. The mixture was kept in a thermostat at 21°, and samples were diluted for assay.

At p_H *8·2.* The procedure was similar to that used above, except that Palitzsch's borate buffer at p_H 8·2 (0·5 ml.) replaced the acetate buffer.

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[1] For definition of this term, see Gulland and Newton [1932].

[2] Assays were carried out by the guinea-pig uterus method, following the "bracketing" technique described by Gulland and Newton [1932]. It should be mentioned that throughout the work described in this paper the response of the isolated uterus was not influenced by the introduction into the testing bath of the reagents or their decomposition products in amounts much greater than those actually used in the experiments. The sole exception to this statement is discussed in the description of the experiments with hydroxylamine.

Iodine (Fig. 4). An aqueous iodine solution at p_{H} 3·5 caused the oxytocic activity to fall to 7 % of its initial value, at which it remained constant.

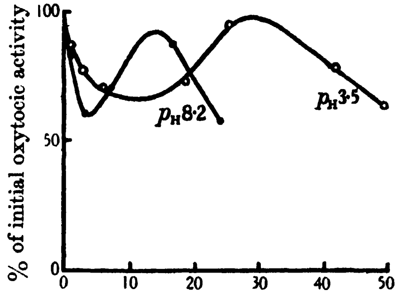

Fig. 3. Action of hydrogen peroxide.

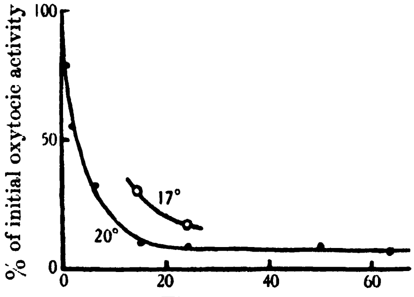

Fig. 4. Action of iodine at p_{H} 3·5.

A mixture of $M/10$ sodium acetate buffer at p_{H} 3·5 (0·5 ml.), aqueous iodine solution saturated at 20° (1·0 ml., 0·28 mg. of iodine) and Kamm solution 1 (0·1 ml.) was diluted to 2·5 ml. and kept in a thermostat at 20°. Samples were suitably diluted for assay. An excess of iodine, as shown by the starch-iodide test, was present throughout the experiments. The temperature coefficient of this reaction appeared to be considerably higher than those of the other reactions which have been studied.

Chlorine. An aqueous chlorine solution completely inactivated the hormone in acid solution in the course of 15 hours at 0°.

A mixture of $M/10$ sodium acetate buffer at p_{H} 3·5 (0·9 ml.), Kamm solution 1 (0·1 ml.) and freshly prepared chlorine water (1·0 ml., 5·6 mg. chlorine) was kept at 0° overnight. A sample was aerated with moist air till free from chlorine (starch-iodide test) and was then adjusted to p_{H} 7 with sodium hydroxide and suitably diluted for assay. The activity of the experimental solution was less than 0·3 % of the initial value.

Semicarbazide had no action on the hormone.

A mixture of a Kamm solution (5·0 ml., containing 10 units associated with 0·1 mg. solid), $M/3$ sodium phosphate buffer at p_{H} 7 (1·0 ml.) and semicarbazide hydrochloride (22·3 mg.) was diluted to 10 ml. at p_{H} 7. The concentration of semicarbazide was thus $M/50$. After the mixture had remained at 0° for 48 hours, the assay of a sample showed that no inactivation had occurred.

Hydroxylamine. The analysis of the effect of hydroxylamine at $M/50$ dilution at p_{H} 7 was not so straightforward as in the case of semicarbazide [*cf.* Gulland, 1933]. In some experiments an apparent inactivation of about 20 % or even 40 % was observed, whereas in others no inactivation could be detected. These discrepancies were finally explained by demonstrating that the responses of some, but not all, uterine horns to a series of standard doses of hormone solution or of histamine were depressed by the simultaneous introduction of hydroxylamine into the testing bath at a dilution of $M/50{,}000$, or sometimes even less. The conclusion was therefore drawn that hydroxylamine has no chemical action on the hormone.

DISCUSSION.

Several of the reagents of which the effects are described in this communication have already been employed by Guha and Chakravorty [1933] and by Das and Guha [1934, 1, 2]. Their results, however, can only be of qualitative value for the following reasons.

In the first place, these authors use the "equality" method of assay; thus Guha and Chakravorty state that "the comparison was made by finding the suitable dose which could produce a contraction in a virgin guinea-pig's uterus equal to that produced by the standard dose", and adherence to this technique is confirmed in the later papers. In our opinion, and we believe that here we share the view of others who have long experience of oxytocic assays, the "equality" method is unreliable and may lead to false conclusions. Its sole recommendation is the fact that the performance of an assay occupies relatively little time and trouble. The only satisfactory method of assay is the "bracketing" method; in this, the least dose of standard clearly greater than the unknown and the greatest dose of standard clearly less than the unknown are ascertained, and the activity of the sample is taken as the mean determined from these values [Burn and Dale, 1922; Burn, 1928; Gulland and Newton, 1932].

Secondly, although Guha and his collaborators recognise the obvious desirability of using hormone solutions which are as free as possible from material which has no oxytocic activity, such, for example, as the preparation of Kamm *et al.* [1928], they do in fact use what must be far less pure solutions of the hormone. There are two objections to the use of hormone solutions which contain much inactive material. In certain reactions the course of the changes undergone by the hormone varies according to the ratio of activity/solid, and in general, the risk is run that the concentration of a reagent may decrease markedly during a reaction owing to preferential interaction with concomitant substances. The consequences are, of course, that the true effect of the reagent on the hormone may not be discerned. Unfortunately, the Indian authors state neither the solid content nor the number of oxytocic units in their solutions, so that exact comparisons are impossible. Nevertheless, to illustrate the point at issue it may be mentioned that in our hands a Kamm solution prepared according to Kamm *et al.* [1928] contained 3·67 mg. of solid (dried at 105° to constant weight) per ml. with which were associated 900 oxytocic units. On the other hand, a solution prepared as described by Guha and Chakravorty [1933] contained 450 units per ml. associated with 28·3 mg. of solid, of which 3·9 mg. were ammonium sulphate.

Thirdly, the practice adopted by Guha and his collaborators of exposing the hormone to the chemical reagent for an arbitrary period of time can only show what the activity is at the end of that period. The observations recorded in this communication alone, to make no mention of the results quoted in previous members of the series, provide ample evidence that experiments made in this way cannot record correctly the progress of a reaction and may give a false impression of its final result. The oxytocic activity must be assayed at suitable intervals of time throughout the reactions.

However desirable it may be to develop at as early a date as possible a distinctive name for the oxytocic hormone, it is unfortunate that Das and Guha [1934, 1, 2] should attempt to designate it as "oxytocin", since this name was given by Messrs Parke, Davis and Co., to one of their commercial preparations of the hormone. It would be regrettable from various standpoints if a chemical entity were to share its name with a commercial preparation, and we prefer to continue to refer to the "oxytocic hormone" until sufficient is known of its properties to permit a rational name to be proposed.

The observations described in this communication cannot be correlated to any degree of probability with a definite structure for the oxidation-reduction systems of the hormone molecule, but it is noteworthy that none is actually opposed to the presence of a disulphide linkage; in fact, several of the results could have been predicted on such an assumption, by analogy with the effects of the same reagents on cystine. Although we do not feel justified in making a definite suggestion that a disulphide linkage is present, it is of interest to compare the action of the reagents on the hormone and on cystine, all the more because Sullivan and Smith [1928] observed that high oxytocic activity of posterior pituitary preparations could be correlated with high content of labile sulphur, and Du Vigneaud *et al.* [1933] found that increasing activity in a series of pitocin (oxytocic hormone) preparations was accompanied by an increase in the sulphur content. The results of these authors may indicate that the sulphur-containing substances from which the hormone is formed are available in larger amounts in active posterior lobes and that the more fully developed forerunners of the hormone molecule resemble it in such a way that their elimination is not readily achieved by processes of purification.

Sodium sulphite and sodium cyanide both inactivate the hormone completely and irreversibly. Their action on cystine is to rupture the disulphide linkage, producing one molecule of cysteine and one molecule of sodium cysteine-*S*-sulphonate [Clarke, 1932; Lugg, 1932] or sodium α-amino-β-sulphocyanopropionate respectively [Pulewka and Winzer, 1928; Lugg, 1933; Bodansky, 1929]. Moreover, Clarke [1932] found that a solution of ammonium sulphite and cystine which was exposed to air ultimately contained cysteine-*S*-sulphonic acid but no cysteine, and he concluded that the cysteine initially formed underwent oxidation to cystine, which in turn reacted with more ammonium sulphite, and so on until the conversion was complete. If the hormone contained a disulphide linkage, analogous changes might lead to disruption of the molecule and consequent total loss of activity with both reagents.

Iodine effected marked inactivation of the hormone, chlorine complete loss of activity. The halogens oxidise cystine to cysteic acid by way of the corresponding sulphinic acid, thus again destroying the disulphide linkage [Shinohara, 1932, 1, 2; Lucas and King, 1932; Simonsen, 1933].

The correlation of the effects of the other reagents with the presence of a specific group is less simple. The successive changes in the activity of the hormone when treated with sulphur dioxide or hydrogen cyanide—decrease, increase and final decrease of activity—might be regarded as evidence for the presence of two oxidation-reduction systems; the reagents could then be regarded as reducing these systems and ultimately as destroying a disulphide-sulphydryl system. Since these reagents have little or no action on cystine, it would be necessary to invoke the well-known lability of sulphur linkages when combined in peptides as compared with the stability in cystine. On the other hand, such arguments lose their value when it is realised that hydrogen peroxide, an oxidising agent, effects the same series of changes—decrease, increase and finally decrease of activity. Hydrogen peroxide oxidises cystine to cysteic acid [Schöberl, 1933].

Finally, it is evident that the oxytocic hormone and insulin exhibit similarities in some of their reactions [see for the insulin reactions: Blatherwick *et al.*, 1927; Freudenberg *et al.*, 1931; 1932; Kuhn *et al.*, 1931; Du Vigneaud *et al.*, 1931–32]. There is, however, one marked difference between these hormones: so far, all attempts to reactivate reduced insulin have proved unsuccessful.

SUMMARY.

1. The action of the following reagents on the hormone has been studied: sodium sulphite, sulphur dioxide, sodium cyanide, hydrogen cyanide, chlorine, iodine, hydrogen peroxide, semicarbazide and hydroxylamine.

2. The results are discussed from the standpoint of the presence of a disulphide linkage in the hormone molecule.

REFERENCES.

Blatherwick, Bischoff, Maxwell, Berger and Sahyun (1927). *J. Biol. Chem.* **72**, 57.
Bodansky (1929). *J. Pharm. Exp. Ther.* **37**, 463.
Burn (1928). Methods of biological assay (Oxford Medical Publications).
—— and Dale (1922). *Med. Res. Coun. Lond. Sp. Rep. Ser.* No. 69.
Clark (1928). Determination of hydrogen ions, 3rd ed. (Baillière, Tindall and Cox, London.)
Clarke (1932). *J. Biol. Chem.* **97**, 235.
Das and Guha (1934, 1). *Indian J. Med. Res.* **21**, 765.
—— —— (1934, 2). *Indian J. Med. Res.* **22**, 157.
Du Vigneaud, Fitch, Pekarek and Lockwood (1931–32). *J. Biol. Chem.* **94**, 233.
—— Sealock, Sifferd, Kamm and Grote (1933). *J. Biol. Chem.* **100**, xciv.
Freudenberg and Dischеrl (1931). *Z. physiol. Chem.* **202**, 193.
—— —— and Eyer (1931). *Z. physiol. Chem.* **202**, 128.
—— and Eyer (1932). *Z. physiol. Chem.* **213**, 226.
Guha and Chakravorty (1933). *Indian J. Med. Res.* **21**, 429.
Gulland (1933). *Chem. and Ind.* **52**, 160.
—— and Newton (1932). *Biochem. J.* **26**, 337,
—— and Randall (1935). *Biochem. J.* **29**, 378.
Kamm, Aldrich, Grote, Rowe and Bugbee (1928). *J. Amer. Chem. Soc.* **50**, 573.
Kuhn, Eyer and Freudenberg (1931). *Z. physiol. Chem.* **202**, 97.
Lucas and King (1932). *Biochem. J.* **26**, 2076.
Lugg (1932). *Biochem. J.* **26**, 2144.
—— (1933). *Biochem. J.* **27**, 668.
Pulewka and Winzer (1928). *Arch. Exp. Path. Pharm.* **138**, 154.
Schöberl (1933). *Z. physiol. Chem.* **216**, 193.
Shinohara (1932, 1). *J. Biol. Chem.* **96**, 285.
—— (1932, 2). *J. Biol. Chem.* **97**, xxii.
Simonsen (1933). *J. Biol. Chem.* **101**, 35.
Sullivan and Smith (1928). *U.S. Pub. Health Rep.* **43**, 1334.
Treadwell and Hall (1928). Analytical chemistry, **2**, 533. (John Wiley and Sons, New York.)

XLVII. CORRELATION OF THE SPERMICIDAL EFFICIENCIES OF AROMATIC ALDEHYDES WITH THEIR CHEMICAL REACTIVITIES, AND ELECTROMETRIC ALKALINE TITRATIONS OF GELATIN IN PRESENCE OF AROMATIC ALDEHYDES.

By JOHN MASSON GULLAND and THOMAS HOBSON MEAD.

From the Biochemical Department, The Lister Institute, London.

(*Received January 2nd, 1935.*)

In a previous communication [Gulland, 1932] it was shown that a series of *p*-benzoquinones arranged in order of their lethal action on guinea-pig spermatozoa resembles closely a series of the same, or similar, quinones placed in order of their chemical reactivities. At the same time the results of a few experiments with aromatic aldehydes were quoted, from which it was evident that certain simple derivatives of benzaldehyde are considerably more effective than others in immobilising guinea-pig spermatozoa. These observations have now been extended to cover a series of closely related aromatic aldehydes, in order to ascertain to what extent their spermicidal efficiency, regarded as a measure of their effect on cells, may be correlated with the chemical reactivities of their aldehyde groups.

The biological tests were made essentially as described by Baker [1930; 1931, 1; 1932] but with one important alteration, which is described in the experimental section. This modification confers the following advantages. (*a*) Substances in pairs may be compared directly with each other, using one and the same sperm suspension. (*b*) Decisive results may be obtained at sublethal, instead of lethal, concentrations, a point which becomes important when sparingly soluble substances are examined. (*c*) It is possible to classify substances which differ but slightly in spermicidal efficiency. (*d*) Fewer animals are needed. The three former advantages were of greater value in the present investigation than in Baker's researches.

The aldehydes now examined were mono- and di-methoxybenzaldehydes; phenolic derivatives were not included, since their acidity disturbed the buffering effect of the fluid in which the sperms were suspended and consequently introduced a second variable factor into the experiments. A method has been devised, however, to enable the spermicidal efficiency of such acidic substances to be tested at the standard p_H value.

The following experimental relationships were established between the lethalities of the aldehydes, formaldehyde being included for comparative purposes: formaldehyde > 2:5-dimethoxybenzaldehyde > 2:3-dimethoxybenzaldehyde > 2-methoxybenzaldehyde ≧ 2:4-dimethoxybenzaldehyde ≧ 3-methoxybenzaldehyde > 3:4-dimethoxybenzaldehyde.

Since the carbonyl group is the only chemically reactive centre in these aldehydes, it is obvious that the amino-groups of the cell proteins or amino-acids are probable points of attack by the aldehydes. It will be shown elsewhere that some aromatic aldehydes condense readily with glycine in presence of baryta or barium acetate, whereas others do not, and in the present connection it may be stated that the isolation of the barium salt of the arylidene glycine was found to be an indication that the aldehyde in question would prove spermicidally efficient. In the same paper it is demonstrated that 2:5- and 2:3-dimethoxybenzaldehydes, both of which are of relatively high lethality towards sperms, enter into reversible condensation with *d*-phenylalanine more rapidly and to a greater extent than does 3:4-dimethoxybenzaldehyde, which has only feeble lethality. Such condensations, however, are less closely related to those occurring in cells than are interactions of the aldehydes with a protein, and the chemical reactivities of the same aldehydes which were examined for spermicidal efficiency have therefore been compared as regards their capacities for condensation with gelatin.

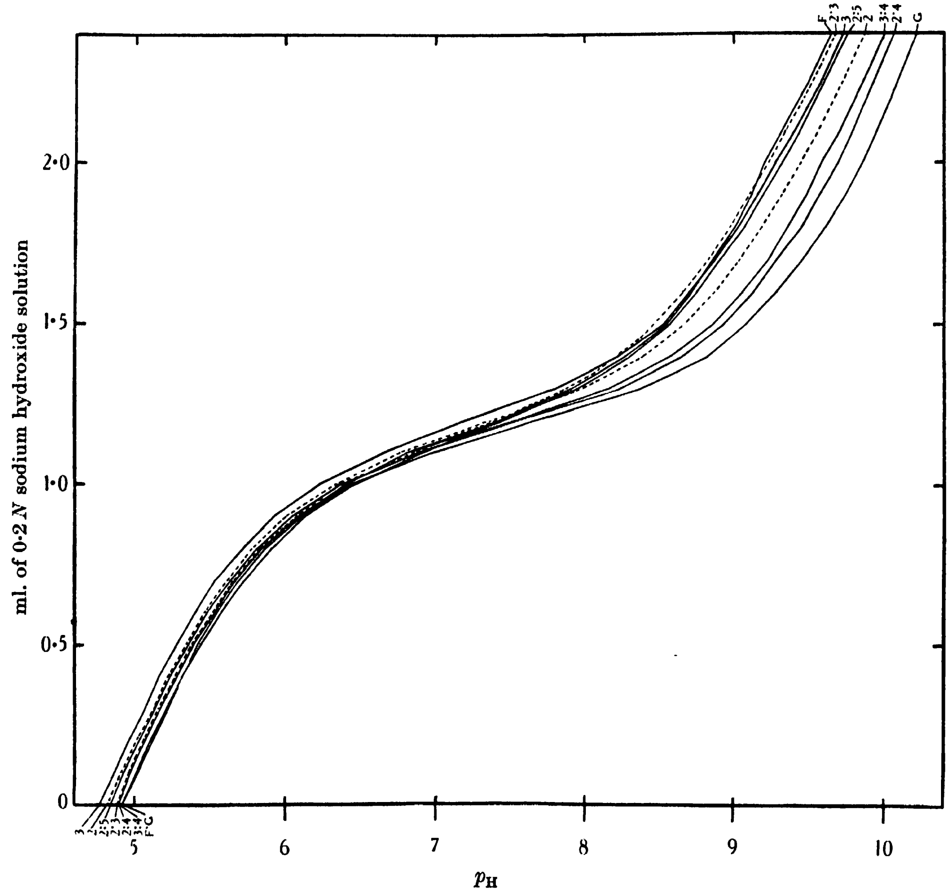

Fig. 1. Titration of gelatin (G) alone and in presence of formaldehyde (F) and 2-, 3-methoxybenzaldehydes, 2:3-, 2:4-, 2:5-, 3:4-dimethoxybenzaldehydes.

Birch and Harris [1930] compared the titration curve of a gelatin solution with that of a similar solution containing formaldehyde and showed that the acidic portion of the curve is unaltered by the addition of the aldehyde whereas in the alkaline range displacement occurs in the direction of decreased p_H value.

This effect is due to the combination of the aldehyde with undissociated basic groups of the gelatin anions. As a result, the effective concentration of gelatin anions is reduced, and the solution becomes more acid [Levy, 1933; Richardson, 1934]. It follows that the extents of the displacements in alkaline titrations of a protein in solutions of different aldehydes at the same concentration provide a measure of the tendencies of the aldehydes to condense with the undissociated amino-groups of the protein anion.

Titration curves were prepared under standard conditions over the range p_H 5–10 for gelatin alone and in presence of a constant fraction of a molecular proportion of each of the aldehydes (Fig. 1). Absolute accuracy was not attempted, but as the conditions of each experiment were identical, the results obtained were strictly comparable among themselves. These curves almost coincide at about p_H 6·5, and closely approach the gelatin curve. They are deflected from the gelatin curve towards increased acidity at higher and lower p_H values (shown more clearly in Fig. 2). These portions of the curves are considered separately below. The curve obtained using 3-methoxybenzaldehyde is apparently abnormal and is discussed later.

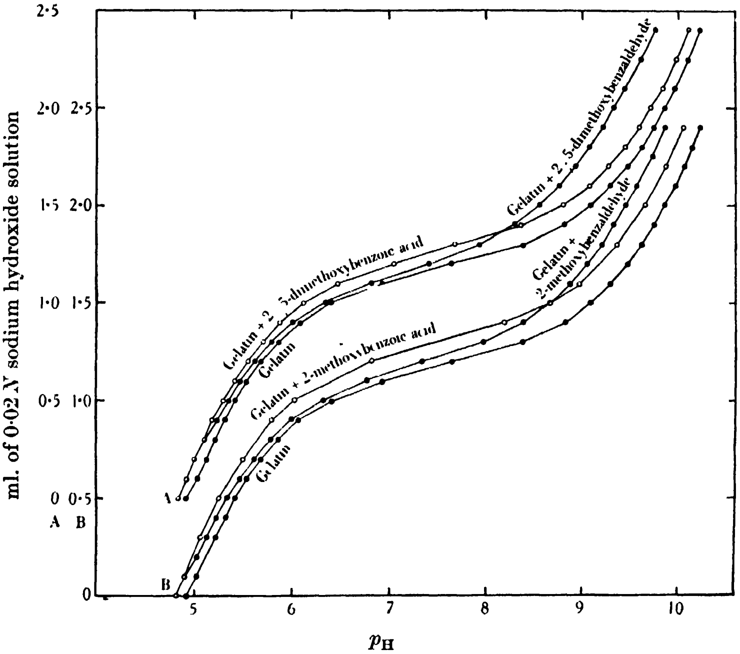

Fig. 2. Titrations of gelatin alone and in presence of:

A { *M*/100 2:5-dimethoxybenzaldehyde
2:5-dimethoxybenzoic acid

B { *M*/100 2-methoxybenzaldehyde
2-methoxybenzoic acid

Consideration of the more alkaline sections of the titration curves shows that condensation occurred progressively over approximately the same p_H range as that in which the amino-groups of the lysine residues and the "terminal" amino-groups of peptide chains dissociate [*cf.* Jordan-Lloyd, 1933]. This suggests that these groups are involved in this condensation.

From the standpoint of the relative capacities of the aldehydes for condensation, the displacements of the titration curves may be compared at p_H 8·2, the reaction of the fluid in which the sperms are suspended in the biological tests, or more easily at $p_{\rm H}$ 9·6, where the spatial differences are greater; the curves do not intersect, with the exception that that of formaldehyde crosses that of 2:3-dimethoxybenzaldehyde at $p_{\rm H}$ 9·1–9·2. By this means the aldehydes were arranged in the following order of decreasing capacity for condensation with gelatin anions: 2:3-dimethoxybenzaldehyde > (formaldehyde) > 3-methoxybenzaldehyde > 2:5-dimethoxybenzaldehyde > 2-methoxybenzaldehyde > 3:4-dimethoxybenzaldehyde > 2:4-dimethoxybenzaldehyde. The position of formaldehyde in the series is doubtful on account of the intersection mentioned above, and 3-methoxybenzaldehyde occupies an unexpectedly high place (see below).

3-Methoxybenzaldehyde autoxidises with great ease, and it was shown by titration that the sample used contained 0·3 % of the corresponding acid, although all possible precautions had been taken to prevent aerial oxidation during its purification. Moreover, the amount of acid may well have increased beyond 0·3 % during the preparation of the aldehyde-gelatin solution before the titration was begun. It was therefore suspected that the position of the titration curve in Fig. 1 was due to the presence of 3-methoxybenzoic acid and not to a superior capacity for condensation by this aldehyde, and confirmation of this view was obtained in two experiments. 2-Methoxybenzoic acid or 2:5-dimethoxybenzoic acid respectively was added to the gelatin solution before titration in amounts sufficient to lower the $p_{\rm H}$ to that of the corresponding aldehyde-gelatin mixture at the isoelectric point of gelatin. The subsequent titration of the mixture of gelatin and acid then followed a course parallel with the gelatin curve and quite different from the aldehyde-gelatin curve (Fig. 2).

By transposing the position of the 3-methoxybenzaldehyde-gelatin curve so that it coincides with the curves of the other aldehyde complexes at $p_{\rm H}$ 6·6, that is, by making allowance for the presence of 3-methoxybenzoic acid, it may be shown that at alkaline reactions 3-methoxybenzaldehyde has a capacity for condensation approximately equal to that of 2-methoxybenzaldehyde, and that it also reacts with gelatin at $p_{\rm H}$ values acid to $p_{\rm H}$ 6·6, in common with the other aromatic aldehydes. These aldehydes thus fall into the following order of decreasing capacity for condensation with gelatin anions at the reaction at which the spermicidal tests were made (Table I). It is also possible that in the spermicidal tests the facile autoxidation of 3-methoxybenzaldehyde reduced its true lethality slightly by decreasing its concentration as a result of conversion into 3-methoxybenzoic acid. Attention was given to these corrections in drawing up Table I, which in our opinion shows the arrangement of the aldehydes in decreasing order (*a*) of spermicidal efficiency, (*b*) of capacity for condensation with gelatin anions.

Table I.

Spermicidal efficiency	Capacity for condensation with gelatin
2:5-Dimethoxybenzaldehyde	2:3-Dimethoxybenzaldehyde
2:3-Dimethoxybenzaldehyde	2:5-Dimethoxybenzaldehyde
2-Methoxybenzaldehyde	2-Methoxybenzaldehyde
3-Methoxybenzaldehyde	3-Methoxybenzaldehyde
2:4-Dimethoxybenzaldehyde	3:4-Dimethoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	2:4-Dimethoxybenzaldehyde

Thus, the series of aldehydes arranged in order of spermicidal efficiency closely resembles the series of the same aldehydes arranged in order of their capacity for condensation with gelatin anions.

The arguments used above are based on the assumption that in no case is the aldehyde present in such amount that all, or even most of those groups in the gelatin molecule which can react have in fact condensed with the aldehyde molecule. That the gelatin was capable of far more extensive condensation was shown (Fig. 3) by the very marked additional displacement of the curve in presence of 0·5 % formaldehyde as compared with that using $M/100$ formaldehyde.

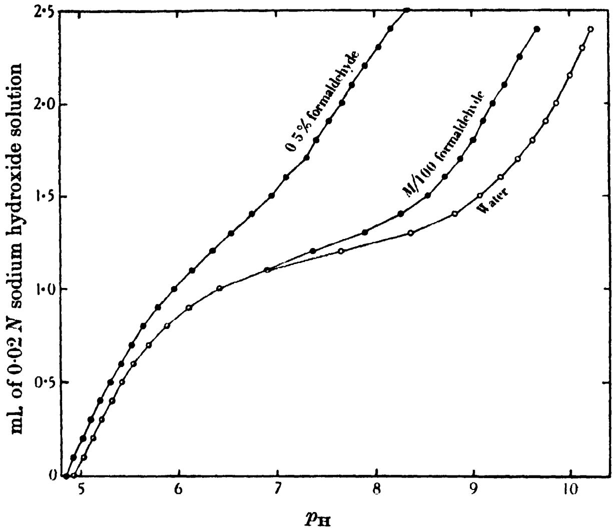

Fig. 3. Titrations of gelatin in 0·5 % and $M/100$ formaldehyde solutions and in water.

The more acidic sections of the titration curves, except in the case of that determined in presence of formaldehyde, are deflected away from the gelatin curve towards greater acidity between p_{H} 6 and 4·9, the isoelectric point of gelatin. These changes are clearly shown in Fig. 2. It is improbable that these deflections are due to the presence of small amounts of the corresponding acids, partly because these aldehydes, excluding 3-methoxybenzaldehyde, do not autoxidise very readily and partly because the curves almost coincide at p_{H} 6·7 as mentioned above. It was shown above that the presence of the corresponding acid prevented this coincidence. It may be inferred, therefore, that the aromatic aldehydes, but possibly not formaldehyde at the same concentration, combine with gelatin between its isoelectric point and about p_{H} 6–7 to form compounds which may be resolved into their components as the reaction of the medium approaches neutrality. This condensation might conceivably be associated with the iminazole group of histidine residues, for which Neuberger [1934] has determined p_{K} 5·99 [*cf.* Jordan-Lloyd, 1933], and in this connection it is interesting to note that when salicylaldehyde and histidine condense in the presence of brucine, the resulting brucine salt contains salicylaldehyde and histidine in the ratio of 2:1 [Bergmann and Zervas, 1926]. We do not, however, advance any explanation of the deflections at acid p_{H} values, since further investigation is necessary.

EXPERIMENTAL.

Technique of biological tests.

The technique described in great detail by Baker showed the least concentration (weight/volume) in the series 1:50, 1:100, 1:200, 1:400, 1:800 *etc.*, which sufficed to immobilise in 30 minutes in four consecutive experiments all guinea-pig sperms suspended in a glucose-saline fluid buffered at p_H 8·1 at 37°. The early experiments of the present investigation were made according to this technique, but it soon became evident that modifications of Baker's procedure were necessary. These are described below, and should be regarded as supplementary to his description and not in any way in opposition to it.

Preparation of buffered glucose-saline solution. It is undesirable to store alkaline stock solution containing glucose and phosphate, owing to the instability of glucose under these conditions. In order, therefore, to avoid the necessity of frequent preparations of small quantities [Baker, 1931, 2] two solutions were employed, one containing the saline constituents, the other the glucose. The first solution consisted of disodium hydrogen phosphate dihydrate (5·96 g.), dihydrogen potassium phosphate (0·20 g.) and sodium chloride (4·00 g.) in 1 litre of aqueous solution; this was preserved at 0° and mixed as required with an equal volume of a solution of glucose (6·0 g.) in freshly prepared aqueous solution (100 ml.). Sodium phosphate dihydrate was used instead of the dodecahydrate [Baker, 1931, 2], since the latter effloresces and is therefore of inconstant composition.

Preparation of aldehyde solutions. These were prepared in terms of molarity, instead of percentage, so that the activities of aldehydes with different molecular weights could be readily compared.

Neutralisation of acidic substances before the biological test. It is well known that sperm-cells are susceptible to changes in the reaction of the medium in which they are suspended, and that the mobility of guinea-pig sperms is much reduced if the medium becomes acid. Measurements of the p_H values of control sperm suspensions made with a B.D.H. capillator before and after a number of experiments showed that an increase in the acidity of the medium from p_H 8·2 to 7·8 might be expected and did not interfere with the mobility of the cells. This change is presumably the result of cell metabolism and respiration and possibly of slight absorption of carbon dioxide from the atmosphere.

Several phenolic aldehydes were examined during this work, and as these substances are acidic, it was possible that part of their spermicidal activities and those of acidic substances in general might be due to insufficient buffering by the glucose-saline medium when the higher concentrations of substances were tested. For example, at the end of an experiment with resorcylaldehyde, the sperm suspension containing this aldehyde at $M/200$ concentration was at p_H 6·8. It was thus essential to neutralise such acidic substances, and this had to be effected without disturbing the isotonicity of the solutions. The following modification of the usual procedure, namely solution of the substance in 0·9 % sodium chloride, was therefore adopted in experiments with acidic compounds. On the assumption that in dilute solution sodium chloride and sodium hydroxide are dissociated to the same extent, an aqueous solution of 6·15 g. of sodium hydroxide in 1 l. was regarded as being isotonic. A solution of the acidic substance (x g.) in 0·9 % sodium chloride (y ml.) at the strength needed for the experiment was titrated with this isotonic sodium hydroxide to p_H 8·0, and the volume of alkali required (z ml.) noted. The solution for the experiment was then prepared by dissolving the substance (x g.) in 0·9 % sodium chloride

(y–z ml.) and isotonic sodium hydroxide (z ml.). This procedure involves the assumption that the sodium salt of the acidic substance was dissociated to the same extent as sodium chloride in dilute solution. This assumption is justified for the purpose of these experiments.

Comparison of biological activities. Baker's technique involves the comparison of the lowest concentrations at which the substances being examined immobilise all sperms under the experimental conditions. There are three objections to this procedure from the standpoint of the present investigation. First, the sparing solubility of several aldehydes prevented lethal or highly active concentrations from being reached. Secondly, the recognition of small differences in activity was extremely difficult, especially when these were encountered in experiments on different days. Thirdly, sperm suspensions vary considerably in the number of cells they contain, and this might affect the results. The following method of performing the tests was therefore adopted.

The effects of two aldehydes, each at two concentrations, were compared simultaneously on one sperm suspension in each experiment. The usual concentrations were $M/200$ and $M/300$, because the stronger solution had some lethal action in each case. Baker's technique was followed closely, with the modifications described. Three slides were used for the microscopic examination, each having two hollows in it, denoted *a* and *b* respectively. Slide I *a* and *b* contained the control suspension. Slide II *a* contained substance x at a dilution of $M/300$, whereas II *b* contained substance y at $M/300$. Slide III contained the two aldehydes at $M/200$ dilution. Thus, by comparing the mobilities of the sperm suspensions *a* and *b* after the standard time of 30 minutes, a direct comparison of the relative activities of the two aldehydes could be made, without relying solely on the records of the grades of activity. Duplicate experiments were made for each pair of aldehydes, and the aldehydes could then be arranged in the order of their activities in immobilising sperms. Quotation of grades of activity recorded and dilutions used in the many experiments would occupy too much space for them to be stated here, although these data were also used in drawing up the series on p. 397. The following list, however, summarises the results of the comparative experiments.

Comparison of the spermicidal efficiencies of pairs of aldehydes.

The activity of:	was	that of:
3-Methoxybenzaldehyde	$<$	2-Methoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	$<$	2:4-Dimethoxybenzaldehyde
2:4-Dimethoxybenzaldehyde	$=$	2-Methoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	$<$	2-Methoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	$<$	3-Methoxybenzaldehyde
3-Methoxybenzaldehyde	$=$	2:4-Dimethoxybenzaldehyde
2-Methoxybenzaldehyde	$<$	2:3-Dimethoxybenzaldehyde
2:3-Dimethoxybenzaldehyde	$\leqq$	2:5-Dimethoxybenzaldehyde
2:3-Dimethoxybenzaldehyde	$<$	Formaldehyde
2:5-Dimethoxybenzaldehyde	$<$	Formaldehyde

Preparation of materials.

Aromatic aldehydes and acids were prepared by customary methods and were purified by repeated recrystallisation, with changes of solvent where possible.

Formaldehyde was prepared by distilling in a slow current of steam a mixture of pure hexamethylenetetramine (14 g.) and dilute sulphuric acid (7 % weight/volume; 300 ml.). Six fractions (40 ml. each) were collected, and the first three (formaldehyde concentration greater than 4 %) were mixed, shaken with calcium carbonate and redistilled in presence of calcium carbonate. The first 40 ml. to be collected were boiled gently in a long-necked flask and cooled *in vacuo*. The residual solution was neutral, was free from carbon dioxide and contained 4·8 % of formaldehyde, estimated by oxidation with neutralised perhydrol in alkaline solution and back-titration of the excess of alkali with standard hydrochloric acid, using phenolphthalein as indicator.

Water free from carbon dioxide was obtained by distilling laboratory distilled water in an apparatus fitted with efficient spray-trap and silica condenser, the first third of the distillate being rejected. The water was collected and stored in a Jena-glass bottle which had been soaked in similar water for some weeks and which was fitted with a soda-lime tube and siphon.

Sodium hydroxide solution. A solution of hydroxide in its own weight of water was left in a resistance glass vessel until the carbonate had separated. The solution was filtered through prepared filter-paper, and diluted with carbon dioxide-free water to give a $0{\cdot}2N$ solution of sodium hydroxide. This solution was preserved in a wax-lined bottle connected to the burette (see later) and protected by a soda-lime tube; its strength was checked at intervals by titration with $0{\cdot}1N$ hydrochloric acid.

Gelatin. Preliminary titration experiments with different samples of "Gold Leaf" gelatin gave inconsistent results, and the gelatin ultimately used was a commercial powder purified as described by Smith [1921]. The final product, a readily soluble powder, was kept in a tightly-stoppered bottle and did not change in composition during the period of the experiments. It contained 9·5 % of moisture when dried to constant weight for 11 hours at 100–105°, and a sample weighing 0·4065 g. left less than 0·1 mg. of residue after ignition.

Purified hydrogen. Hydrogen was obtained from a cylinder connected with a small gas-holder which helped to maintain a steady flow. The gas was scrubbed by passing up two towers, the first filled with glass beads over which flowed concentrated sodium hydroxide, the second filled with beads over which passed water free from carbon dioxide. Traces of oxygen were removed by passing the hydrogen over copper gauze heated at a dull red heat in an electric furnace.

Electrometric titrations of gelatin alone and in presence of aldehydes.

Apparatus. The use of rubber tubing in conveying the hydrogen supply was avoided as far as possible, connections between glass tubes being arranged so that the tubes met in the joint. All rubber stoppers and tubes were purified by treatment with sodium hydroxide and water in the usual way.

The cell consisted of a pyrex boiling-tube, 5 cm. in diameter and 12·5 cm. long, having a working capacity of 100 ml. The tube was closed by a rubber bung pierced with seven holes, through which passed a glass stirrer in a tight sleeve, a thermometer, inlet and exit tubes for hydrogen, and closely fitting glass sleeves through which passed the tip of the burette, the agar potassium chloride bridge and the hydrogen electrode respectively. These sleeves enabled the easy insertion or withdrawal of the parts in question. The stirrer was operated manually and could be raised to remove drops adhering to the tip of the burette.

The hydrogen electrode vessel was of the Bunker type [1920], so designed that the entire platinum surface (about 1 cm. of wire) was bathed in hydrogen as each bubble escaped, and was then covered with liquid before the next bubble formed. The electrodes were replatinised before each titration, and occasionally a fresh electrode was inserted during a titration in order to make certain that poisoning was not occurring. After a suitable interval for saturation, the fresh electrode was always at the same potential as that which it had replaced.

The reference electrode was a 3·5N potassium chloride-calomel half-cell connected to the hydrogen electrode cell by a vessel containing saturated potassium chloride and the agar-potassium chloride bridge. Fresh surfaces of the bridges were exposed before use, and bridges gave concordant results when interchanged.

The electrodes were connected to a Tinsley potentiometer by unsupported wires, and the null point was determined by a capillary electrometer. Two determinations of E.M.F. were made, the null point being approached from both sides.

Before reaching the cell, the hydrogen passed through a presaturator containing pure water for all experiments except those with solutions containing formaldehyde, when the presaturator contained a formaldehyde solution of the same strength as that in the cell. Condensation in the tube between the presaturator and the cell was prevented by an adjacent carbon-filament lamp.

The cell, presaturator, saturated potassium chloride vessel and calomel electrode were immersed in a paraffin thermostat at 37° ± 0·1°.

The burette was protected by soda-lime tubes and was fitted with two jets, one which could be lowered into the cell, the other arranged outside the thermostat for adjusting the zero level and for checking the strength of the sodium hydroxide.

Procedure. Purified gelatin (1 g.) and the aldehyde (0·1 millimol) in water (100 ml.) were used for each experiment, except in one instance when more concentrated formaldehyde solution was used. The total volume of 0·2N sodium hydroxide required in each titration was 2·4 ml., and the range p_H 5–10 was covered. The aldehyde and gelatin were either dissolved simultaneously by warming their suspension in the water to 90° and then cooling to 38°, or the gelatin was dissolved first in this way and the aldehyde was added to the warm solution. The results were not affected by these changes in procedure, and the latter alternative was adopted in the cases of 3-methoxybenzaldehyde and formaldehyde.

The calomel electrode and potassium chloride vessel were placed in the thermostat 30 minutes earlier than the cell, the contents of which were saturated by a slow stream of hydrogen through the inlet tube. Ten minutes later, the tip of the burette was arranged to deliver just above the surface of the liquid in the cell, the hydrogen electrode and its vessel were inserted, and the hydrogen supply was diverted from the inlet tube to the electrode. Twenty minutes later the agar-bridge was inserted, and equilibrium had usually been attained after a further period of 10 minutes.

When the E.M.F. was constant, the titration was begun by the addition of sodium hydroxide solution in portions of 0·1 ml., the new equilibrium being reached within 2 minutes. The contents of the cell were stirred after each addition and between each measurement of potential. Steady values were obtained between p_H 5–6 and 8–10, but occasionally drifts towards the acid side were observed near the neutral point. Since these were observed to the same extent with gelatin alone, it was concluded that they were not due to increased time taken for the aldehyde-gelatin system to reach the new equilibrium. An accuracy of 0·01 p_H unit is probable for the values obtained in the more acid and alkaline regions, but this accuracy is slightly less in the case of values about the neutral point on account of the drifts of potential.

The p_H values were calculated from the observed potential differences by the Nernst expression for the E.M.F. of a concentration cell.

In the titrations of gelatin in presence of small amounts of aromatic acids, a 0·02N solution of the required acid was added to the gelatin solution in the cell until the p_H value had fallen to the same extent as had been observed in titrations of gelatin-aldehyde mixtures where small amounts of the corresponding acid were suspected. The titration of the acid-gelatin mixture was then carried out with 0·02N sodium hydroxide in the usual way. In these experiments 4·2 mg. of 2:5-dimethoxybenzoic acid and 3·6 mg. of 2-methoxybenzoic acid were used.

SUMMARY.

1. A method is described for comparing directly on the same material the spermicidal efficiencies of two substances. This is specially valuable when sparingly soluble compounds are to be examined.

2. This method has been used to arrange a series of mono- and di-methoxybenzaldehydes in the order of their spermicidal efficiencies.

3. Titration curves of gelatin in presence of the same mono- and di-methoxybenzaldehydes are deflected from the gelatin curve towards a more acid reaction over the range p_H 8–10. This deflection is a measure of the extent to which the aldehydes have condensed at equilibrium with the gelatin anion between these p_H values.

4. A series of mono- and di-methoxybenzaldehydes arranged in order of their capacities for condensation with the gelatin anion closely resembles the series of the same aldehydes arranged in order of their spermicidal efficiencies.

5. Titration curves of gelatin in presence of mono- and di-methoxybenzaldehyde are deflected from the gelatin curve towards a more acid reaction between the isoelectric point and p_H 6–7.

6. A method is described for testing the spermicidal efficiencies of acidic substances which disturb the buffer effect of the media in which the sperms are suspended.

REFERENCES.

Baker (1930). *J. Hyg.* **29**, 323.
—— (1931, 1). *J. Hyg.* **31**, 189.
—— (1931, 2). *Quart. J. Exp. Physiol.* **21**, 139.
—— (1932). *J. Hyg.* **32**, 171.
Bergmann and Zervas (1926). *Z. physiol. Chem.* **152**, 282.
Birch and Harris (1930). *Biochem. J.* **24**, 1080.
Bunker (1920). *J. Biol. Chem.* **41**, 11.
Gulland (1932). *Biochem. J.* **26**, 32.
Jordan-Lloyd (1933). *J. Internat. Soc. Leather Trades' Chemists*, 245.
Levy (1933). *J. Biol. Chem.* **99**, 767.
Neuberger (1934). *Proc. Roy. Soc. Lond.* B **115**, 180.
Richardson (1934). *Proc. Roy. Soc. Lond.* B **115**, 121.
Smith (1921). *J. Amer. Chem. Soc.* **43**, 1350.

XLVIII. AN ULTRACENTRIFUGAL INVESTIGATION OF THE SERUM PROTEINS.

By ARTHUR SPROUL McFARLANE.[1]

From the Institute of Physical Chemistry, The University, Upsala, Sweden.

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The extensive researches into the molecular dimensions of the proteins carried out in recent years by Svedberg and collaborators[2] by means of the ultracentrifuge have hitherto been conducted mainly on dilute protein solutions. This has been necessitated by the fact that the sedimentation velocity in the centrifugal field is independent of the protein concentration only in dilute solution. In addition to this, various practical difficulties arise when it is desired to examine protein solutions, such as serum, which contain more than 1–2 % of protein. For instance, the light absorption in the ultraviolet of such solutions is generally too great to permit the use of the absorption method of following the protein sedimentation. Also, in the case of serum, the absorption over the whole cell would not be due to the proteins alone, as is desirable, but also to the serum pigments.

The refractive index method of Lamm [1928; 1929], which depends on photographic measurements of the light deviation caused by the sedimenting boundary, offers better possibilities of examining strong protein solutions, because in this method light may be used of a wave-length which is outside the absorption region of the proteins or of any non-centrifugible absorbing substances which may be present in the solution. Mutzenbecher has recently used this method extensively in his studies on the serum proteins [1933, 1, 2, 3]. In this method a regular ruled scale is photographed through the centrifuge cell as it rotates, and according to whether the sedimentation boundary is sharp or diffuse the scale must be placed very close to, or at some distance from, the centre of the centrifuge cell. It is at present mechanically impossible to bring the scale closer to the centre of the rotating cell than 3·5 cm.,[3] and at this distance the maximum concentration of serum albumin which can be examined in the thinnest centrifuge cell available is about 4 %, and of globulin about 2 %, owing to its lower diffusion constant. In the course of experiments directed towards the design of a system which might permit satisfactory centrifugal examination of undiluted human serum it was suggested to the writer by Dr Lamm that in place of the actual scale it should be possible to project optically close to, or even inside, the rotating centrifuge cell an image of an actual scale placed at some distance from the centrifuge. This scheme was immediately successful and afforded for the first time excellent photographs of the sedimentation of the proteins in undiluted serum and in some artificial protein mixtures of even higher concentration. It was found, for instance, that with certain undiluted sera scale distances as small as 0·9 cm. were required to give successful photographs. It was also found, however, that there is a definite limit to the concentration of protein which may be examined owing to the appearance at high concentrations of a black band across the scale which completely blots out the lines in the boundary area. This phenomenon, which apparently arises when the deviation of the beam is so great that the light from the mercury lamp fails to reach the camera at all, must depend on a great variety of factors for its appearance, such as the thickness of the cell, the refractive gradient in the solution and the relative positions of lamp, cell, objective lens and camera. The phenomenon is closely related to the "Totalreflexion" described by Gans [1915] in his studies on the propagation of light through inhomogeneous media. On the basis of our experience with the present optical system of the ultracentrifuge we may say that the complication does not in general occur with serum protein concentrations less than 10 % in a 1·5 mm. cell, and that higher concentrations may usually be examined unless there is an abnormally large proportion of globulin or substances of higher molecular weight. With globulin alone, the black band will appear first at a concentration of approximately 6 %.

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[1] Beit Memorial Research Fellow.

[2] A complete bibliography of this literature will be found in a recent article by Svedberg [1934].

[3] In the new rotors which are in process of construction it will be possible to bring the scale to within 2·5 cm. of the centre of the cell.

The method would appear to open a new field, *viz.* that of very concentrated protein solutions, and promises therefore to throw light on the molecular properties of many proteins in their natural biological state. There will be reported here the results of an investigation into some new phenomena which appear in concentrated mixtures of albumin and globulin, and which affect fundamentally our present ideas of the molecular state of these proteins in untreated serum. Throughout the work the opportunity has been taken to verify experimentally some of Lamm's more fundamental equations relating to the deviation of light by diffusing systems.

GENERAL THEORETICAL CONSIDERATIONS.

(*a*) *The refractive index method of Lamm.* A full account of the theory of the refractive index method will be found in Lamm's original papers. As already stated, a ruled scale is photographed through the centrifuge cell as it rotates and those lines on the scale which are seen through the diffusing boundary area appear in displaced positions. A photograph obtained in a preliminary run at the same speed but with water only in the cell gives a standard undeviated scale in which, of course, the effect of the compression gradient throughout the fluid in the cell is present and may be seen by comparing the average distance between the lines with the same distance on a photograph taken at low speeds. During the subsequent run with the protein solution in the cell photographs are taken at intervals of 10–20 mins. and the position of each scale line on the photograph is then measured with an accuracy of $\pm 1\mu$ in a microscope comparator. In order to find the deviation (Z) of any line in a photograph from its position in the standard undeviated scale it is necessary to have the same comparator zero for all photographs. On each scale photograph there appear also 3 or 4 scale lines which are photographed directly through small apertures in the balancing cell of the centrifuge. Since the absolute positions of these lines in space remain unaltered during the run, one of these is chosen as reference zero and every scale is placed on the microscope stage so that the arbitrary comparator reading for this line is 0.

The photographic displacement of any line (Z) from its position on the undeviated scale is given by

$$Z = G\,a\,b\,\frac{dn}{dx} \qquad \text{......(1)}$$

where G = the photographic enlargement factor;

a = the thickness of the fluid layer in the cell traversed by the beam;

b = the optical distance from the scale to the middle of the cell;

and $\frac{dn}{dx}$ is the gradient of refractive index, along a radial axis, at the displaced line position in the plane of the cell.

The photographic displacement (Z) for each line is plotted against its displaced position in comparator units. In this way a curve is obtained which shows the change of refraction gradient throughout the cell (*cf.* Fig. 2). If we assume that the gradient is zero in those parts of the cell above and below the diffusion region (which is usually experimentally true) then the area enclosed by this curve and the base-line is given by

$$A=\int_{-\infty}^{+\infty}\left(G\,a\,b\,\frac{dn}{dx}\right)dx=G\,a\,b\,(n_1-n_0) \qquad \ldots\ldots(2),$$

where n_0 is the refractive index of the solution of non-sedimenting electrolytes which separates above and n_1 is the refractive index of the protein solution below the sedimenting boundary.

It is to be emphasised that in the derivation of this equation no assumptions are made as to the nature of the forces which give rise to the gradient, and in particular, that the validity of the formula is not dependent on the curve conforming to any particular type.

The area A, obtained by graphical integration, is multiplied by the following factors:

1. $\left(\frac{x}{x_0}\right)^2$ where x and x_0 are the distances of the ideal sedimentation boundary (*i.e.* the theoretical boundary assuming that no diffusion has taken place) and the meniscus respectively from the centre of rotation. This factor [Svedberg and Rinde, 1924] corrects for the sector shape of the cell and the fact that the protein is sedimenting in a centrifugal field the intensity of which varies with the distance from the centre of rotation.

2. A factor (F) which converts the arbitrary comparator and graph units into absolute units (cm.).

3. $\frac{l}{(l+b)\,G}$ where l is the distance of the camera objective lens from the centre of the cell. This factor converts the displaced line positions on the photograph into absolute positions in the plane of the cell.

The equation
$$n_1-n_0=\frac{A}{G\,a\,b}\cdot\left[\frac{x}{x_0}\right]^2\cdot\frac{l}{(l+b)\,G}\cdot F \qquad \ldots\ldots(3)$$

gives, therefore, the protein refraction increment of the solution, but on the condition already stated, *viz.* that the solution which separates above is free from centrifugible matter and that no high molecular protein is thrown to the bottom of the cell before the first photograph is taken. Such an assumption is, of course, only justifiable for approximately monodisperse protein solutions and in particular for protein solutions which are free from protein disintegration products.

(*b*) *The calculation of sedimentation velocity.* The sedimentation constant is given by

$$S=\frac{dx}{dt}\cdot\frac{1}{x_m\omega^2} \qquad \ldots\ldots(4),$$

where the boundary has moved through a cell depth dx in time dt under a mean centrifugal force of $x_m\omega^2$. In every case we have corrected this value to a medium of pure water at 20° by means of the formula

$$S_{\mathrm{W},20}=S_{\mathrm{S},\mathrm{T}}\cdot\left[\frac{\eta_{\mathrm{S},\mathrm{T}}}{\eta_{\mathrm{W},20}}\right]\left[\frac{\left(\frac{1}{V}-\rho_{\mathrm{W},20}\right)}{\left(\frac{1}{V}-\rho_{\mathrm{S},\mathrm{T}}\right)}\right] \qquad \ldots\ldots(5),$$

where $S_{W,20}$ is the sedimentation constant in water at 20°;
$S_{S,T}$ is the sedimentation constant in the salt solution at $T°$;
$\eta_{W,20}$ is the viscosity of water at 20°;
$\eta_{S,T}$ is the viscosity of the salt solution at $T°$;
V is the partial specific volume of the protein (=0·745 for horse serum albumin and globulin [Svedberg and Sjögren, 1928]);
$\rho_{S,T}$ is the density of the salt solution at $T°$;
$\rho_{W,20}$ is the density of water at 20°.

Tiselius [1932] has considered the electroviscous effect which appears when charged particles are caused to move in relation to the surrounding fluid by centrifugal forces and which in certain circumstances may give rise to abnormally small values of S. He gives the approximate formula

$$S^1 = S\,(1 - t_k) \qquad \text{......(6)},$$

where S^1 is the apparent sedimentation constant, S is the true sedimentation constant, and t_k is that fraction of the total conductivity (colloid+all other electrolytes) which is due to the colloidal ions. t_k varies widely with the protein, salt and hydrogen ion concentrations. In spite of the unusually high protein concentrations which we have examined in this work the value of t_k is always small because of the high conductivity of the 1 % sodium chloride or Ringer solution used as dispersion medium for the protein. No electroviscous correction need therefore be applied to the sedimentation constant values.

(c) *The base-line of the sedimentation diagram.* A phenomenon of rather frequent occurrence in the sedimentation graphs consists in a shift of the apparent base-line of the curve, *i.e.* the line joining the two horizontal limbs, so that it lies above or below the true base-line or x-axis. It appears that a displaced base-line is always associated with the use of a large centrifuge cell or a large scale distance or the two together. With water alone in the cell it may be seen from the scale photographs that the light beam passing through the cell is deviated as a whole (in relation to the undeviated beam passing through the apertures in the balancing cell) even at low speeds, and that the angle of deviation changes progressively with changing speed of the rotor. The amount of deviation is too great to be due to the compression gradient in the water. It is possible that the deviation is caused by a lack of true parallelism in the quartz end-plates of the cell. A consideration of the cell as a prismatic deviator of small angle shows that the deviation will increase with an increase of a or b, and this has been observed in practice. It is also possible that changes in the quartz plates under the influence of the high centrifugal forces used may contribute in large part to the deviation.

Pedersen [1934] has found that this deviation is strictly reproducible in the same cell when great care is taken to maintain the speed of the rotor constant to within ± 100 r.p.m. When this and other precautions which we need not mention here are observed it is possible to determine the true base-line, as Pedersen has done in his sedimentation equilibrium experiments with inorganic salts. In a prolonged series of experiments such as we are concerned with here, and in which it is proposed to examine a wide range of concentrations, it is not possible to use the same cell throughout and to observe all the precautions which Pedersen has been able to take. It will be apparent that if an insidious shift of the base-line is allowed to occur, then the area enclosed by the curve and the true base-line, or x-axis, will be abnormal and a serious error will be introduced in the calculations of $n_1 - n_0$ (equation (3)). If instead we measure the area enclosed by the curve and the apparent base-line this will still be accurately related to $n_1 - n_0$ in accordance with the terms of equation (3), provided that by $n_1 - n_0$ we understand the difference in the refractive indices at two levels in the cell just outside the area of the diffusing boundary—one above and one below, and not necessarily as in the original definition, the difference between the refractive indices of the protein solution and of the salt solution in which the protein is dispersed. We have, therefore, throughout this work adopted the convenient and safe procedure of basing all measurements on the apparent base-line. It should, however, be pointed out that in thus abandoning temporarily the true base-line we have no longer an absolute reference point for our optical measurements, and in particular, that it is impossible to make measurements either of absolute refractive index or of absolute refraction gradient in the cell. This means that we cannot compare directly the refractive index of the solution which separates above with that of the inorganic solution in which the protein is dispersed, which we would wish to do in order to verify the absence of so-called non-centrifugible matter. Neither is it possible to compare the refractive index of the solution below the boundary with that of the original protein solution in order to verify the absence of any molecules which might be sedimenting in advance of the protein. In both these cases, however, it is very unlikely that such material would be present in the cell without giving rise to irregularities on the base-line of the curve or without causing it to slope. We have indeed found, especially with globulin solutions, base-line irregularities of this nature, and we have always been able to confirm indirectly the presence of contaminating matter in these cases by comparing the calculated value of $n_1 - n_0$ with the measured value of the total protein refraction increment after dialysis. The difference between the two represents the amount of colloidal matter which is retained in the dialyser and which nevertheless does not appear in the apparent area of the diffusion curve.

The error involved in the sedimentation velocity measurements in using the apparent base-line instead of the true base-line is negligible.

(*d*) *The measurement of refraction increment.* Before each experiment the protein solution is dialysed against 1 % sodium chloride or Ringer solution until ionic equilibrium is reached, and the refractive indices of the solution and its dialysate are then measured. With the high concentrations of protein which we have used, and notwithstanding the relatively high concentration of diffusible electrolytes, considerable inequality in the distribution of diffusible ions is present at equilibrium such as is capable of giving rise to appreciable osmotic pressure effects. Since, however, the refractivity of one molecule of albumin or globulin is so much greater than the corresponding value for sodium chloride (more than 1000 times) the effect on the refraction increment is very small. Adair and Robinson [1930, 1], in a series of experiments on horse serum albumin using concentrations up to 10 g. per 100 ml. of solution in $M/15$ phosphate buffer at p_H 7·4, found the effect of the uneven distribution of diffusible ions to be within the limits of their experimental error, *viz.* 3 %. We have always used higher concentrations of diffusible electrolytes than $M/15$ and p_H values usually less than 7·4, so that the effect in our case must be even smaller. In calculating protein concentrations from these refractometric measurements we have used Adair and Robinson's values for the specific refraction increments of horse serum albumin and globulin, *viz.* 0·00183 and 0·00186 respectively. These are corrected values and we have not endeavoured to apply to our measurements any corrections for Donnan effect or the volume of the protein molecules. Our concentration values may therefore be in error, but by not more than 3 %. For the total protein of horse serum we have assumed a mean value, *viz.* 0·001845.

In the process of sedimentation a protein boundary is set up at which the same uneven distribution of electrolytes must exist, and which, so far as we know, is subject in every way to the same Donnan considerations as the boundary at the collodion bag in the preliminary dialysis [*cf.* Tiselius, 1926]. We are, therefore, strictly justified, subject to certain considerations governing the conditions under which the two measurements are made, in comparing directly the refraction increment from measurements of the refractive indices of the solution and its dialysate with the value obtained from the sedimentation diagram.

With regard to the conditions of measurement it will be seen that the value for $n_1 - n_0$ which is measured with the refractometer is for a wave-length in the region of $590 m\mu$ and at room temperature and atmospheric pressure. From the ultracentrifugal data, on the other hand, we compute $n_1 - n_0$ for the $366 m\mu$ mercury line, at a cell temperature which varies between 30° and 36° and under a hydrostatic pressure of up to 300 atmospheres. The writer has measured the refraction increment of serum albumin over the range 15° to 30° and has found the maximum change to be less than 1 %, so that this factor can be neglected. The pressure factor may be neglected also since the hydrostatic pressure inside the centrifuge cell varies from about 300 atmospheres at the bottom to practically zero at the meniscus, and there is no evidence of the calculated values for $n_1 - n_0$ being significantly different at the bottom of the cell and at the top. It is not possible to neglect the wave-length factor, however. Pedersen and Andersson have recently undertaken an investigation of the variation of the specific refraction increments of various proteins and inorganic salts with the wave-length of the light used, and I am greatly indebted to them for permission to give here some of their preliminary results. They have used a differential prism apparatus arranged for photographic registration of the undeviated and refracted beams. The values in Table I are calculated relative to Adair and Robinson's values for horse serum albumin and globulin. They were obtained in the examination of various preparations of horse serum proteins made by Pedersen and McFarlane according to the technique which will be described later in this paper. Some of the albumin preparations had been electrodialysed and even dried and redissolved, but the agreement between all values for the same wave-length was always within 1 %. The solutions were examined at 20° with the dialysate (water, 1 % sodium chloride or Ringer solution) in the other compartment of the prism. These experiments of Pedersen and Andersson are still in progress and may lead to the publication of new absolute values for α_{albumin} and α_{globulin}, but there is no reason to expect that further investigation will lead to any change in the relative values at different wave-lengths, and in this particular case it is only the relative values which matter. It will be seen that the value for α at $366 m\mu$ is greater than the value at $589 m\mu$ by 0·00015 in the case of albumin, and 0·00016 in the case of globulin. We have,

Table I. *The specific refraction increments* (α) *of horse serum albumin and globulin at different wave-lengths* (*Pedersen and Andersson*).

Wave-length $m\mu$	α_{albumin}	α_{globulin}
366	0·00198	0·00202
436	0·00190	0·00195
546	0·00185	0·00187
579	0·00183	0·00186
589	0·00183	0·00186
656	0·00179	0·00182

therefore, corrected all our values for $n_1 - n_0$ obtained by measurements either with the dipping refractometer, or the Pulfrich refractometer using the sodium D line, to the wave-length 366 mμ by means of the following relationship:

$$n_1 - n_0 \text{ (measured at } 590\,m\mu) = [n_1 - n_0 \text{ (measured at } 366\,m\mu)] \times 1{\cdot}08.$$

This holds with sufficient accuracy both for horse serum albumin and globulin, and we assume it to hold also for the total protein of horse serum.

It would, of course, have been a more direct procedure to use light of the same wave-length for measuring the refractive indices of solution and dialysate and for photographing the scale. Unfortunately, the technique for measuring refractive index in invisible, or poorly visible, regions of the spectrum is not nearly so convenient as with easily visible light, and on the other hand, the line at 366 mμ is much to be preferred for taking centrifuge photographs. It is conveniently present in the mercury arc spectrum in great intensity and almost monochromatic filters are available for its isolation; it is not absorbed to any appreciable degree by most proteins, and it lies in a spectral region in which photographic plates are highly sensitive. While it is possible to use lines in the green or red regions, in general the photographs with these do not show the same degree of contrast as with the mercury line at 366 mμ, and we have, therefore, preferred to use this line throughout and to apply the correction factor given above.

(e) *The measurement of "diffusion" constants.* The diffusion constant may be calculated in various ways from measurements taken from the sedimentation diffusion curve [Lamm, 1928; 1929]. There are, however, a number of factors which reduce materially the accuracy with which diffusion coefficients may be thus obtained from centrifugal data. The exact time at which the diffusion process starts is not known and is almost certainly variable between experiments according to the degree of convectional disturbance at the meniscus during the initial acceleration period. Whereas in an ideal diffusion experiment with proteins extending usually over a number of days it is possible to eliminate the unknown starting time between the equations of two exposures [*e.g.* see Tiselius and Gross, 1934], this cannot be done here with any degree of accuracy because of the very short time during which the diffusion process may proceed and because the diffusing boundary is sedimenting in a cell the walls of which are not parallel. Also, it is not possible to have the temperature of the diffusing solution the same in all experiments. Finally, in the case of protein solutions more concentrated than about 0·5 % the sedimentation velocity is markedly influenced by the protein concentration. This means that as the protein molecules at the boundary diffuse into the supernatant fluid they enter a region of diminished protein concentration in which their sedimentation velocity is increased. The effect of the centrifugal force, therefore, is to cause a "sharpening" of the boundary in opposition to the natural process of diffusion. This sharpening effect will be slightly greater at the bottom of the cell than at the top owing to the increase in sedimentation velocity as we proceed away from the centre of rotation. As will be shown later, it is even possible, with undiluted sera and concentrated globulin solutions, to have the diffusion process so completely counteracted by the differential sedimentation that the boundary sediments to the bottom of the cell with an apparent decrease in the breadth of the diffusion area.

Even a very approximate idea of the extent of "spreading" or "blurring" of the boundary may be of some value, however. Where we are concerned with a mixture of two or more proteins differing so little in molecular weight that a separation of the maxima cannot be obtained with the centrifugal forces available, or with a protein contaminated with numerous molecules slightly larger and slightly smaller than its own (a state of affairs which we have reason to believe exists in certain circumstances), then the only evidence that we are not dealing with a monodisperse protein lies in an abnormal spreading of the boundary. We have therefore endeavoured in this work to calculate a value for this in each case. Now it is clear from the foregoing that we are concerned with a composite effect in which the most that may be said meantime is that the process of diffusion is, in general, a major influence. For this reason, and in the absence of any complete theoretical treatment of the phenomenon, we propose to apply to our concentrated solutions the recognised methods of calculating diffusion coefficients in dilute solutions in the centrifuge cell and to call the resulting figure the spreading coefficient. In doing so we wish it to be understood that spreading coefficients are purely arbitrary measures of the apparent diffusion at a boundary which is sedimenting under non-ideal conditions, and that they may only be compared with each other under strictly comparable conditions of sedimentation. In a private communication Lamm has given the following equation for an ideal diffusion process

$$K.t=\frac{A^2}{Z^2_{\max}}\cdot\frac{1}{4\pi} \qquad \text{......(7)},$$

where K is the diffusion coefficient, t is the time of diffusion, A is the area of the diffusion curve at time t, and $Z_{\text{max.}}$ is the height of the curve. Therefore, for any two exposures, at times t_a and t_b, we have

$$K(t_a-t_b)=\frac{1}{4\pi}\left[\frac{A_a^2}{Z^2_{a\max}}-\frac{A_b^2}{Z^2_{b\max}}\right] \qquad \text{......(8)}.$$

This equation is used throughout to calculate the spreading coefficient. The exposures are chosen as far as possible the second from the beginning and the second from the end of the run. Errors arising in the calculation of individual boundary areas may give rise to large errors in the spreading coefficient because in equation (5) we are concerned with the difference between the squares of the areas. Now the value of each boundary area multiplied by Svedberg and Rinde's factor $(x/x_0)^2$ [1924] is a constant independent of the time at which the exposure was taken. We have, therefore, avoided the above source of error by obtaining firstly an average value for $A\,(x/x_0)^2$ over all exposures and dividing this by $(x_a/x_0)^2$ and $(x_b/x_0)^2$ to obtain the values for A_a and A_b respectively. The x values are usually obtainable with a much higher degree of accuracy than the A values.

GENERAL EXPERIMENTAL TECHNIQUE.

Fig. 1 shows schematically the optical system fitted to the high-speed oil turbine centrifuge which was used throughout this work. The scale and its associated projection lens are mounted together in fixed relationship on a metal base which slides on an optical bench. The position, in relation to a fixed point on the metal base, and the size of the scale image were first determined by photographic means using the mercury line at 366$m\mu$ and allowing for the presence of the quartz windows in the centrifuge housing, but neglecting the effect of the rarified hydrogen atmosphere inside the centrifuge. The optical bench was then graduated in terms of the distance from the scale image to the mid-plane of the rotor (or centre of cell). By means of a fine screw adjustment the scale-lens combination could be set at any position on the bench and the corresponding distance from image to centre of cell read off to 0·1 mm. In addition to enabling much smaller scale distances to be used, this arrangement offers a number of valuable practical advantages over the original method in which the scale was fixed inside a steel tube in the centrifuge housing. For instance, in the course of an experiment using the refraction method the scale-lens combination may be temporarily removed from the optical path and a light-absorption exposure taken in order to measure the concentration distribution of any light-absorbing substance in the cell. Also, since the scale position can be altered during an experiment it is not necessary to know beforehand the value of b which, in association with the boundary gradient to be expected in the cell, will give scale photographs with the requisite degree of definition. The determination of this scale position was formerly a matter requiring some experience. Now it is only necessary to view the scale from the camera by means of a hand lens immediately before the first photograph is to be taken and to judge with the eye which scale position gives the maximum line displacement in the diffusion area consistent with good definition of the displaced lines. Where a mixture of two or more proteins is under examination, one of which is present in much smaller amount than the other, it is also possible, when sedimentation has proceeded long enough to effect a clear separation of the two diffusion boundaries, to photograph the line displacement due to each boundary separately, using the best scale position for each photograph. In this way, where mixtures are concerned, the analytical sensitivity of the method may be increased many times.

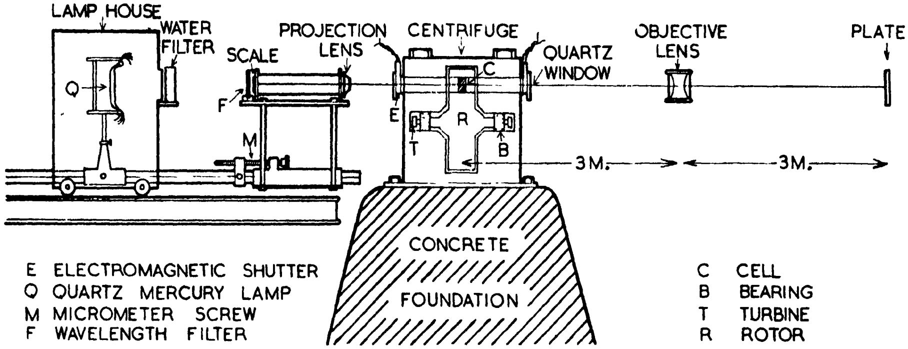

Fig. 1. Showing diagrammatically the new optical system fitted to the high-speed oil turbine centrifuge, which was used throughout this work.

Throughout the work to be described in this and following papers efforts were made to employ as small a number of centrifuge cells as possible and to run the centrifuge at a speed in the neighbourhood of 60,000 r.p.m., which corresponds to a mean force of 255,000 times gravity in the cell. In a preliminary run at 60,000 r.p.m. with each cell filled with water two photographs were obtained for each of a series of scale positions, 3 mm. between each—one just after starting the centrifuge and the other on reaching full speed. The latter served as the standard undeviated scale for that particular scale position and cell, and the former was used to obtain the photographic enlargement factor. The mercury line at $366 m\mu$ was used in all cases and was isolated by means of a nickel oxide glass filter. Imperial Process plates were used in the earlier work but later plates made by G. Cramer and Co., St Louis, Mo., U.S.A., were used, which, although requiring longer exposures, had the advantage of giving better contrast.

According to the concentration of protein, a period of 1–3 hours after reaching 60,000 r.p.m. was required to produce complete sedimentation of albumin and globulin. During this time six to eight photographs were taken at equally spaced intervals and with suitably chosen stops over the objective lens according to the degree of definition of the displaced scale lines. The temperature inside the cell at reaching full speed was usually 28–32° and did not rise by more than 4° during the whole run. The height of the column of solution in the cell was 10–11 mm. and the solution was always covered by a layer of paraffin to prevent evaporation into the rarified hydrogen atmosphere in the centrifuge housing.

The general plan of the investigation was to prepare and centrifuge separately solutions of purified albumin and globulin in various concentrations and then to examine a series of mixtures of the two in various proportions. The preliminary refractive index measurements after dialysis were made at first with the Zeiss dipping refractometer, but subsequently with the Pulfrich refractometer using the "difference" prism and the sodium D line as light source. The two methods of measurement are in essential agreement here but the latter is more convenient in practice, and in later work with highly coloured untreated serum, the use of monochromatic light was found strictly desirable. The hydrogen ion concentrations were measured by means of the glass electrode using a valve potentiometer already described by the writer [1932; 1933, 1, 2]. A few experiments—particularly with dilute solutions –in which the apparent base-line did not run parallel to the x-axis, or the horizontal limbs of the curve differed markedly in height above the x-axis (for reasons probably associated with the establishment of convection gradients in the cell), were discarded and are not reported. But, for the great majority of runs, the apparent base-line was satisfactory and enabled an accurate determination of A and the position of the maximum (corresponding to the position of the "ideal" sedimentation boundary) to be made. In the case of mixtures of two proteins the component curve with the most clearly defined maximum was completed by symmetry first and the other constructed by a process of graphical subtraction from the original.

Methods of preparing serum proteins.

Preparation of horse serum albumin. To fresh horse serum an equal volume of saturated ammonium sulphate was added, and after standing overnight the supernatant fluid was filtered off, treated with $N/10$ sulphuric acid in half-saturated ammonium sulphate to slight permanent cloudiness and given 24 hours to crystallise. The crystals were dissolved in water and recrystallisation induced by half-saturation with ammonium sulphate and careful addition of M acetic acid and M sodium acetate according to the procedure described by Young [1922]. The preparation was recrystallised four times in all and washed between each crystallisation with a solution of approximately the same inorganic composition as the mother-liquor. The final aqueous solution was dialysed and then electrodialysed at 440 v. until the current density was steady at 0·5 ma. per sq. cm. Finally the solution was made quite clear by centrifuging in an ordinary laboratory centrifuge at 2000 r.p.m. and filled into a number of small sterile bottles. The solution had a pale amber colour and was quite odourless.

Preparation of horse serum globulin. The globulin precipitate (above) was dissolved from the filter-paper with water and reprecipitated by half-saturating the solution with ammonium sulphate. The precipitate was washed with half-saturated ammonium sulphate. The precipitation and washing were repeated three times and the final solution dialysed against frequent changes of Ringer solution and under pressure until the product was quite free of sulphate ions and had a concentration of approximately 5 % globulin. This solution was centrifuged clear and stored in the same way as the albumin. The solution was pale blue, faintly opalescent and also quite odourless.

DESCRIPTION AND RESULTS OF A TYPICAL EXPERIMENT.

A solution of horse serum albumin after prolonged dialysis against Ringer had a refractive index of 1·34193, and the dialysate 1·33500. Therefore

$$n_1 - n_0 = 6{\cdot}93 \,.\, 10^{-3},$$

and the concentration of albumin is $\frac{0{\cdot}00693}{0{\cdot}00183}$, or 3·79 g. per 100 ml. solution. The p_H of solution (measured) = 5·26. The solution was placed in a centrifuge cell of thickness (a) 1·5 mm. and with quartz end plates 5 mm. thick. The scale distance was 4·0 cm. Refractive index of crystalline quartz = 1·56.

b (optical distance from scale to centre of cell)

$$= 4{\cdot}0 - \left(0{\cdot}5 + \frac{0{\cdot}15}{2}\right) + \frac{0{\cdot}5}{1{\cdot}56} + \frac{0{\cdot}15}{2 \times 1{\cdot}34} = +3{\cdot}80 \text{ cm.}$$

The centrifuge was started and reached 60,000 r.p.m. in 30 mins. The first exposure was taken 20 mins. later and thereafter five additional exposures were taken at intervals of approximately 10 mins. The scale photograph of each exposure was then measured in the comparator, the line displacements were obtained by subtraction, and the curve showing photographic displacement of each line against its displaced position was plotted. Figs. 2 *a* and 2 *b* show the curves corresponding to the second and fifth exposures. The high degree of symmetry of these curves and the almost complete absence of irregularities of the base-line testify in large measure to the monodispersity of the serum

Table II. *Results of a typical experiment with horse serum albumin.*

$G = 0{\cdot}981$. $\frac{l}{(l+b)} = 0{\cdot}987$. $x_0 = 6{\cdot}266$ cm. $F = 1 \,.\, 10^{-4}$.

Exposure no.	Time (mins.)	x cm.	$(x'/x_0)^2$	A sq. cm.	$(n_1 - n_0) \,.\, 10^3$	$S_{W,20} \,.\, 10^{13}$ cm./sec.
1	0	6·421	1·050	35·30	6·63*	—
2	10	6·482	1·070	32·77	6·27	3·18
3	20	6·533	1·093	31·88	6·23	3·58
4	30 -	6·627	1·119	31·23	6·25	3·66
5	40	6·717	1·149	30·14	6·19	3·57
6	51	6·778	1·170	29·73	6·22	3·44

Average value (excluding the first) of $(n_1 - n_0)$ $= 6{\cdot}23 \,.\, 10^{-3}$,
Average value (excluding the first) of $S_{W,20}$ $= 3{\cdot}56 \,.\, 10^{-13}$ cm./sec.,
Spreading coeff. (calculated between Exps. 2 and 5) $= 1{\cdot}6 \,.\, 10^{-7}$ cm.^{2}/sec.

* An abnormally large area in the first exposure occurred frequently throughout the present series of experiments. It may be due to optical effects in the neighbourhood of the meniscus, or to the fact that separation from the meniscus was not complete when the photograph was taken. On the other hand, a more probable explanation is that the preparation contains some polydisperse protein molecules which at the beginning of sedimentation give rise to a relatively sharp boundary, but in the later stages the molecules are so separated by the centrifugal field as to give rise to a very diffuse boundary the curve of which merges with the apparent base-line. While it would be of some interest to know in each experiment the maximum area of the sedimentation curve in the early part of the run, we cannot record this because we do not know when to take the photograph, and also very early photographs are often impossible because the gradient at the meniscus is so great. We must, therefore, confine our measurements to those exposures for which the values of the protein refraction increment are constant, and in the process of averaging the refraction increment values for one complete experiment we have accordingly omitted the value for the first exposure where this was clearly abnormal.

albumin preparation. The abscissa of the vertical axis of symmetry of each curve was read off and from it the distance (x) of the boundary from the centre of rotation was calculated. The distance (x_0) of the meniscus from the centre of rotation was similarly calculated and the value of the sedimentation constant obtained by means of equations (4) and (5). Finally, the area of each curve on the graph paper was obtained by counting the equivalent millimetre squares and the value of $n_1 - n_0$ calculated using equation (3). Table II shows the results of this experiment.

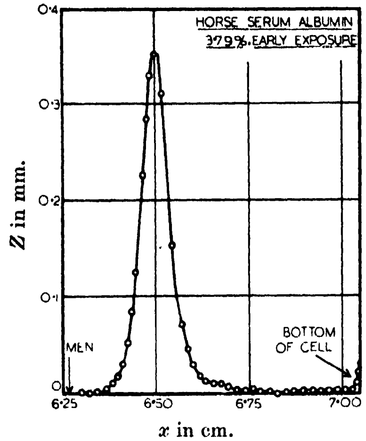

Fig. 2 (a). $a = 1{\cdot}50$ mm.; $b = 3{\cdot}80$ cm. Centrifugal force = 255,000 times gravity. Exposure taken 30 mins. after reaching full speed.

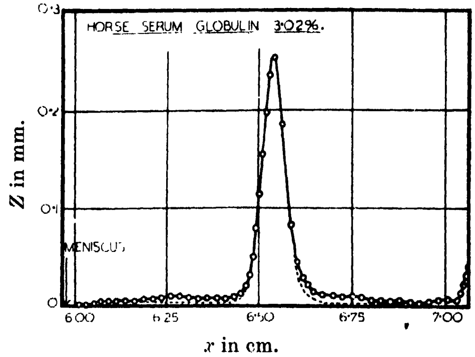

Fig. 2 (c). $a = 2{\cdot}00$ mm.; $b = 2{\cdot}41$ cm. Centrifugal force = 210,000 times gravity. Exposure taken 46 mins. after reaching full speed.

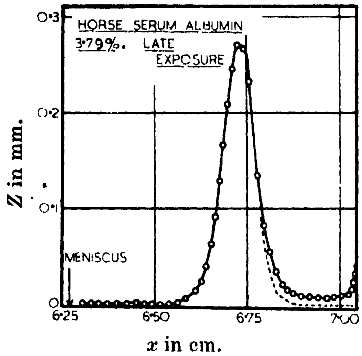

Fig. 2 (b). From same experiment as (a). Exposure taken 60 mins. after reaching full speed.

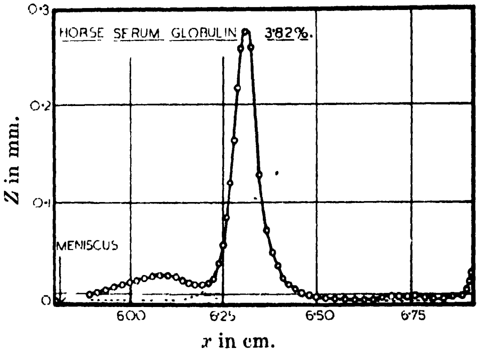

Fig. 2 (d). $a = 2{\cdot}00$ mm.; $b = 2{\cdot}56$ cm. Centrifugal force = 250,000 times gravity. Exposure taken 55 mins. after reaching full speed.

Fig. 2. *Ultracentrifugal sedimentation diagrams obtained by the refraction method.* The abscissae (x) represent distances from the centre of rotation, and the ordinates (Z) represent the scale-line displacements which are proportional to the gradient of refractive index (dn/dx). A comparison of (a) and (b), from the same experiment, shows how the curve moves parallel with the x-axis during the run, and also shows the symmetry and regularity typical of the albumin curves. A comparison of (c) and (d) shows the differences which may be expected in the sedimentation diagrams of different globulin preparations.

DISCUSSIONS OF RESULTS OBTAINED WITH ALBUMIN AND GLOBULIN SOLUTIONS.

In Table III are presented the results obtained from the investigation of various horse serum albumin and globulin solutions. Preparations A2, A3, A4 are all dilutions of the same stock solution of albumin, and the curves all show the same degree of symmetry and absence of base-line irregularities as are shown in Figs. 2 *a* and 2 *b* (from preparation A2). Preparation A1 is also from the same stock solution which, in this case, had been preserved for 6 months in the electrolyte-free state and adjusted to 1 % NaCl just before the experiment. Slight base-line irregularities were present here, suggesting the presence of about 2 % of larger molecules. Also, the value of the sedimentation constant is unexpectedly high. In Fig. 2 *c* is shown one of the curves obtained in the investigation of the globulin preparation G4. The irregularity of the base-line of this curve was also present in the curves of G2, G3, G5 and G6, which are all dilutions of the same stock solution. By constructing the symmetrical curve (shown in dotted lines) it is estimated in this case that the preparation is only 90 % monodisperse, the remaining 10 % consisting of molecules both larger and smaller than the principal molecule. Preparation G1 is from a different stock solution prepared in exactly the same way as the stock solutions of G2, G3 *etc.*, but from a different horse serum. Fig. 2 *d* shows a late exposure from this experiment and it will be seen that the preparation is contaminated with about 20 % of a lighter molecule, presumably albumin. There seems to be no explanation of this other than a possible difference in the constitution of the parent horse serum in the two cases. Data to be presented in a subsequent paper relative to a variety of globulin preparations from human, horse and cow serum will show these preparations to be contaminated to a variable extent with larger or smaller molecules. It does not seem to be possible, merely by a process of repeated precipitation and washing, to obtain a globulin preparation with the degree of monodispersity shown by recrystallised albumin. This observation is one of considerable significance because there has been in the past, and still exists, considerable variation in the physico-chemical properties of serum globulin as recorded by different observers—particularly in regard to solubility, osmotic pressure and isoelectric point. In so far as the methods adopted to measure

Table III. *Results obtained in a series of experiments with solutions of horse serum albumin and globulin.*

Protein solution	Protein conc. g./100 ml.	Medium	p_H	Cell thickness mm.	Optical scale dist. cm.	Average $S_{w,20}.10^{13}$ cm./sec.	Measured $(n_1-n_0).10^3$ $\lambda=366m\mu$ (*a*)	Calculated average $(n_1-n_0).10^3.\lambda=366m\mu$			"% diff." $\frac{(a-b)}{a}\times 100$	Boundary spreading coefficient $\times 10^7$ cm.²/sec
								Principal molecule	Other molecules	Total (*b*)		
Albumin experiments:												
A 1	5·40	1 % NaCl	—	2·00	1·96	3·80	10·7	8·62	0·16	8·78	−17·9	1·3
A 2	3·79	Ringer	5·26	1·50	3·80	3·56	7·48	6·23	0	6·23	−16·7	1·6
A 3	0·47	,,	5·16	6·00	7·57	4·56	0·93	0·81	0	0·81	−12·9	7·4
A 4	0·173	,,	6·40	12·0	9·0	4·60	0·349	0·305	0	0·305	−12·6	8·3
Globulin experiments:												
G 1	3·82	1 % NaCl	—	2·00	2·56	5·83	7·68	5·42	1·15	6·57	−14·5	0·1
G 2	3·02	,,	6·51	2·00	2·41	6·44	6·07	5·10	0·25	5·35	−11·9	0·2
G 3	2·45	Ringer	6·51	1·50	3·80	6·27	4·91	4·10	0·30	4·40	−10·4	1·7
G 4	1·48	,,	6·54	1·50	3·80	6·69	2·97	2·16	0·22	2·38	−19·9	5·2
G 5	0·52	,,	6·51	4·0	11·6	6·82	1·05	0·82	0·04	0·86	−18·1	8·6
G 6	0·174	,,	6·54	12·0	11·5	7·20	0·349	0·250	0·040	0·290	−16·9	7·6

these properties are only capable of giving a statistical mean result for all the molecules present, the lack of agreement among different observers may be explained by variations in the composition of different globulin preparations, such as we have found here by ultracentrifugal analysis.

The marked diminution of sedimentation constant with increasing concentration of albumin and globulin will be at once evident. Also at globulin concentrations greater than 3 % practically no active process of spreading at the boundary can be detected.

The column of Table III headed "% diff." expresses the difference between the measured and calculated values of $n_1 - n_0$ as a percentage of the measured values. These values show an average loss of approximately 15 % over all the experiments, and a variation of individual values about the mean "% diff." of never more than ±5 %. This systematic discrepancy of −15 % is difficult to explain. It may be due to basing all our calculations on the apparent instead of the true base-line. If so, it is probable that all our albumin and globulin preparations contain about 15 % of protein which is so completely heterodisperse as not to affect the base-line of the curve. Another source of error, but only in the experiments with the smaller cells, lies in the fact that we cannot measure the thickness of the cell to less than 0·1 mm. because periodical tightening of the assembly rings, which is necessary to prevent leakage, causes small changes in the distance apart of the plates, and also the plates are not absolutely parallel. Changes in the refractive index of the quartz plates under high pressure may be a contributory cause. It is conceivable, also, that the sedimenting assymetrical protein molecules are orientated in a particular plane in relation to the light beam and thus show an abnormal refractivity.

More important, however, than the extent of the discrepancy between measured and calculated $n_1 - n_0$ is the fact that the error appears to be almost entirely systematic and reproducible. It is considered that the agreement of the individual results among themselves, extending, as the experiments do, over a wide range of protein concentration, affords strong support for the validity of Lamm's fundamental equations and demonstrates the practicability of estimating protein concentrations from the scale photographs. When one considers the practical difficulties which beset these measurements of protein refraction increment, using 0·1–0·5 ml. of solution inside an optically imperfect cell and rotating at a speed of 60,000 r.p.m., the whole being observed from a minimum distance of 6 metres, it will be appreciated that the figure of ±5 % for the error in individual experiments is a very satisfactory one. When, further, one considers the possibilities which are presented to estimate the molecular fractions in untreated native protein mixtures and mixtures of non-absorbing substances, even with an error of ±5 %, it will be realised also what considerable satisfaction this substantiation of Lamm's equations gives rise to.

EXPERIMENTS WITH MIXTURES OF ALBUMIN AND GLOBULIN.

The general scheme underlying the preparation and investigation of these mixtures was as follows. A fresh horse serum (undiluted horse serum 1) was examined in the ultracentrifuge without previous treatment of any kind. The curve obtained (Fig. 3 *a*) showed the presence of two molecules corresponding in sedimentation constants approximately to albumin and globulin. The "albumin" component curve is unsymmetrical and represents in its total area 79·1 % of the total protein. The "globulin" curve, representing 20·9 %, is quite sym-

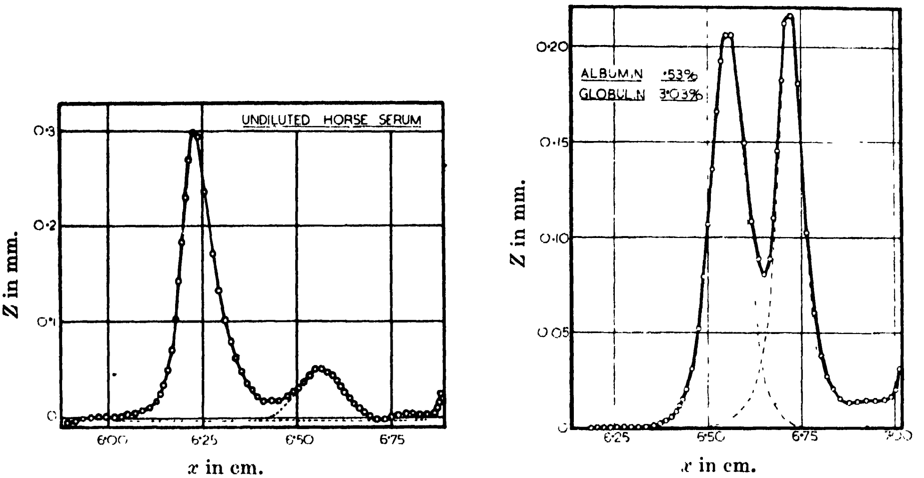

Fig. 3 (*a*). $a = 2{\cdot}00$ mm.; $b = 2{\cdot}14$ cm. Centrifugal force = 235,000 times gravity. Exposure taken 117 mins. after reaching full speed.

Fig. 3 (*c*). $a = 2{\cdot}00$ mm.; $b = 3{\cdot}62$ cm. Centrifugal force = 250,000 times gravity. Exposure taken 57 mins. after reaching full speed.

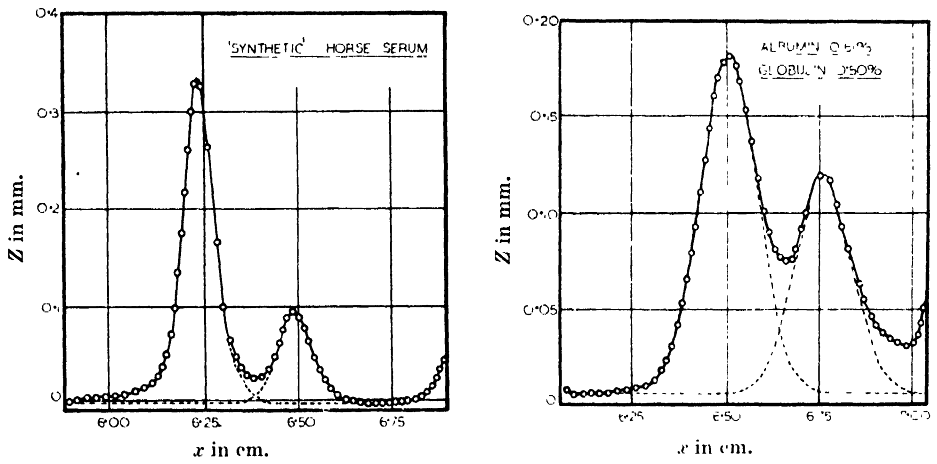

Fig. 3 (*b*). $a = 2{\cdot}00$ mm.; $b = 2{\cdot}26$ cm. Centrifugal force = 250,000 times gravity. Exposure taken 92 mins. after reaching full speed.

Fig. 3 (*d*). $a = 3{\cdot}0$ mm.; $b = 11{\cdot}10$ cm. Centrifugal force = 235,000 times gravity. Exposure taken 60 mins. after reaching full speed.

Fig. 3. *Ultracentrifugal sedimentation diagrams by the refraction method showing the dilution effect in serum and albumin-globulin mixtures.* (*a*) shows the surprisingly small amount of "globulin" present in undiluted horse serum; (*b*) is from an experiment using a mixture of albumin and globulin, both prepared from the serum used in (*a*) and subjected to the usual purification procedures. They were mixed together in the same ratio and approximately the same concentration as in the original serum; (*c*) is from mixture 6, containing 1·53 g. albumin/100 ml. and 3·03 g. globulin/100 ml.; and (*d*) is from mixture 4, containing 0·61 g. albumin/100 ml. and 0·50 g. globulin/100 ml.

metrical, and the base-line is free from irregularities. An analysis of the serum by precipitation with ammonium sulphate gave 51 % albumin and 49 % globulin. The original serum was then used to prepare recrystallised albumin and reprecipitated globulin by methods identical with those already described

(p. 416), and a mixture of the two preparations in the same ratio and of very roughly the same total protein concentration as in the original serum was made up ("synthetic" horse serum 1). Here again the proportion of "albumin" was found to be abnormally high (72·7 %) (*cf.* Fig. 3 *b*). One or two tentative experiments were now tried to suggest a reason for the abnormal albumin/globulin ratio and in one of these the original serum was examined after dilution with 3 volumes of 1 % NaCl. It was noticed that the ratio had now fallen very considerably. Mixtures of albumin and globulin covering a wide range of concentrations were then made up using the same stock albumin and globulin solutions as had been used for A2, A3, A4, and G2, G3, G4, G5 and G6 (Table III) and the behaviour of which on dilution had, therefore, been examined in the ultracentrifuge. The results are shown in Table IV.

Discussion of results.

(*a*) *Sedimentation constants.* The values for $S_{W,20}$ for the "albumin" fraction show a marked concentration effect similar to that shown by pure serum albumin. Also, if we plot the "albumin" values against the calculated value of n_1-n_0 (column D), and show on the same diagram the same values for pure albumin (from Table III) it appears within the rather wide limits of comparability that the sedimentation velocity of the "albumin" fraction in a concentrated mixture is the same as the sedimentation velocity of a pure solution of albumin of the same concentration. In the case of the "globulin" values a marked concentration effect is present, and also the values from the concentrated mixtures as a whole are uniformly smaller than the values for the same concentration of globulin in pure solution. It is impossible to say whether this effect is entirely due to the viscosity and density of the albumin solution in which the globulin molecules are sedimenting, or whether any additional changes in the shape and dimensions of the "globulin" molecule have occurred. It is only at concentrations less than 0·5 % of each that the two proteins in a mixture appear to sediment independently of each other.

(*b*) *Refraction increment.* The figures in the column headed "'% diff.' (total n_1-n_0)" represent the difference between the measured and calculated values of the total protein refraction increment of the mixture, expressed again as a percentage of the former. The average difference is −12·4 %, and the individual figures may be represented by the approximate formula

$$n_1-n_0\ (\text{measured})=n_1-n_0\ (\text{calculated})-(13\ \%\pm 9\ \%).$$

The systematic error agrees well with the value found in the experiments reported in Table III. The range of individual variation in the mixtures is, however, greater than in the case of the pure solutions. This is probably due to the greater difficulty in locating accurately the apparent base-line where, as in the mixtures, only comparatively short horizontal portions of curve can be seen. It is to be noted also that, in spite of the fact that the original globulin of these mixtures is only approximately 90 % monodisperse, the mixtures appear practically free from base-line irregularities (*cf.* Figs. 3 *c* and 3 *d*). No explanation can be offered of this. In view of the agreement between the average "% diff." values of Tables III and IV we conclude that when albumin and globulin are mixed together the total concentration of the added proteins is represented by the sum of the areas of the component curves in the diagram of the mixture. This simple conclusion assumes its full significance when we consider the refractive equivalents of the individual components in the diagram of the mixture.

Table IV. *Results obtained in the investigation of horse serum and mixtures of horse serum albumin and globulin.*

Protein solution	Protein conc. g./100 ml.	Medium	p_H	Cell thickness mm.	Optical scale distance cm.	Average $S_{W.20}.10^{13}$ cm./sec.	Measured $(n_1-n_0).10^3$ $\lambda=366 m\mu$ Individual	Measured $(n_1-n_0).10^3$ $\lambda=366 m\mu$ Total (*a*)	Calculated average $(n_1-n_0).10^3$ $\lambda=366 m\mu$ Individual (*d*)	Calculated average $(n_1-n_0).10^3$ $\lambda=366 m\mu$ Total (*b*)	"% diff." total (n_1-n_0) $\left(\frac{a-b}{a}\right)\times 100$	Theoretical expected individual (n_1-n_0) (*c*)	"% diff." individual (n_1-n_0) $\left(\frac{c-d}{c}\right)\times 100$	Boundary spreading coefficient $\times 10^7$ cm.²/sec.
Undiluted horse serum	A 3·75 G 3·50	—	—	2·00	2·11	2·94 4·35	7·42 7·04	14·46	10·43 2·75	13·18	− 8·9	6·76 6·42	+54.3 −57·2	0·8 2·6
'Synthetic' horse serum	A 4·09 G 3·82	1 % NaCl	—	2·00	2·26	3·34 4·72	8·09 7·68	15·77	9·95 3·74	13·69	−13·2	7·02 6·67	+41·8 −43·9	0·5 3·1
1	A 2·04 G 2·00	Ringer	5·79	2·00	3·62	3·72 5·49	4·03 4·03	8·06	4·21 2·27	6·48	−19·6	3·24 3·24	+30·0 −30·0	2·0 1·9
2	A 1·02 G 1·00	*M*/2·6 acetate buffer	4·67	3·00	5·00	4·12 5·87	2·02 2·02	4·04	2·56 1·29	3·85	− 4·7	1·92 1·92	+33·3 −33·3	5·1 3·7
3	A 1·02 G 1·06	*M*/6·7 phosphate buffer	7·61	3·00	5·00	4·16 6·38	2·02 2·13	4·15	2·18 1·32	3·50	−15·7	1·70 1·80	−28·2 −36·3	4·0 2·0
4	A 0·61 G 0·50	Ringer	5·72	3·00	11·1	4·43 6·63	1·21 1·01	2·22	1·27 0·86	2·13	− 4·1	1·16 0·97	+ 9·48 −11·3	7·7 5·0
5	A 0·127 G 0·125	"	6·40	12·0	11·5	4·81 7·31	0·250 0·250	0·50	0·218 0·210	0·428	−14·0	0·215 0·215	+ 1·87 − 1·87	9·1 7·4
6	A 1·53 G 3·03	"	6·42	2·00	3·62	3·77 5·14	3·02 6·09	9·11	4·08 3·50	7·58	−16·8	2·52 5·07	− 62·6 − 44·8	2·4 1·3
7	A 2·15 G 1·26	"	5·51	1·50	3·80	3·79 6·18	4·24 2·53	6·77	3·91 1·39	5·30	−21·7	3·32 1·98	+ 17·8 −29·8	2·1 5·0
8	A 0·48 G 0·23	"	5·96	3·00	11·6	4·57 6·75	0·95 0·47	1·42	0·98 0·379	1·36	− 1·2	0·91 0·45	+ 7·7 −15·6	7·8 4·8

A = Albumin. G = Globulin.

It is at once apparent in all the more concentrated mixtures that the concentration of the "globulin" component is much less than corresponds to the globulin added, and that the concentration of the "albumin" is correspondingly much greater. In an attempt to express equitably the relative loss of globulin and increase of albumin we have firstly divided the discrepancy between measured and calculated total $n_1 - n_0$ between the two components in the ratio in which they were originally added together and thus we obtain a "theoretically expected" value for the refraction equivalent of the area of each component curve in the diagram (*cf.* column headed "theoretically expected" individual $n_1 - n_0$). The difference between this value and the value calculated for the refraction equivalent of the actual area of the component curve, expressed as a percentage of the former, is shown under "'% diff.' (individual $n_1 - n_0$)". A consideration of the figures in this column leads to the following general conclusions. The "albumin"/"globulin" ratio is always shifted in the direction of increased "albumin". The extent of shift increases markedly with increasing concentration of total protein and is particularly marked with high relative concentrations of globulin (*cf.* mixture 6, Fig. 3 *c*). The phenomenon seems to appear only at concentrations greater than 0·25 % total protein. From a comparison of mixtures 2 and 3 it is deduced that hydrogen ion concentration and the nature of the inorganic ions present seem to have little, if any, effect. The results suffice to demonstrate the existence in mixtures of albumin and globulin of a type of molecular dimensional equilibrium the nature of which is primarily dependent on the concentrations in which the two proteins are present. Before discussing some theoretical aspects of the phenomenon we may consider briefly the boundary spreading coefficients shown also in Table IV.

(*c*) *Boundary spreading coefficients.* In Fig. 4 we have plotted the value of the spreading coefficients of the "albumin" boundaries in each mixture against the calculated values of $n_1 - n_0$ for the same fraction. A mean line has been drawn through the points and its course demonstrates the marked suppression of boundary diffusion which takes place at high protein concentrations and under the roughly standardised sedimentation conditions of our experiments. We have also shown on the same diagram as ringed points the values from Table III for the four albumin solutions, and it will be seen that these points lie tolerably well on the curve. It appears, therefore, that the degree of spreading at the boundary of the "albumin" fraction in a mixture is not significantly greater than the degree of spreading at a sedimenting boundary of pure albumin

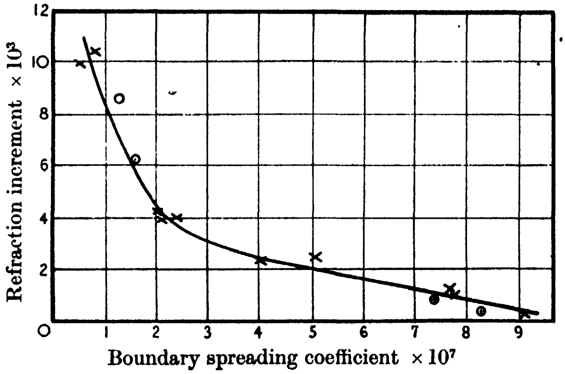

Fig. 4.

of the same concentration. This we consider to be strong contributory evidence in favour of the "albumin" fraction in a mixture being dimensionally homogeneous. We can say nothing with regard to the homogeneity of the globulin fractions because the spreading coefficients cannot be compared with each other owing to the variable effect of the albumin concentration.

GENERAL DISCUSSION.

In the first place we may summarise many of the above data by the statement that only when the total protein concentration is less than 0·25 % does a solution of albumin and globulin behave ideally. At all higher concentrations the diffusion, sedimentation and relative concentration of the component proteins are abnormal.

Throughout this work we have referred consistently, when speaking of the mixtures, to the "albumin" and "globulin" components. This terminology is merely a matter of convenience since the available data are insufficient to define accurately the true nature of either molecular fraction. It can be said from a consideration of the sedimentation constants that the average molecular weight of the lighter fraction corresponds in all mixtures approximately to that of albumin, and from considerations of the degree of boundary spreading, that the fraction is probably homogeneous. On the other hand, in concentrated mixtures it is present in much greater amount than the monodisperse albumin which may be obtained from the same mixture by treatment with ammonium sulphate. With regard to the heavier fraction we can only say that in diluted mixtures it has all the characteristics of salted-out globulin and in concentrated mixtures it may still be the same substance. In the latter case, however, it is present in amount much less than the practically monodisperse globulin which may be obtained by precipitation.

The question has often been asked whether in a solution of two proteins of different isoelectric points mutual neutralisation of the electrical charges on the colloidal particles may not take place, such as occurs with lyophobic colloids of opposite charge and leads to precipitation. Hardy [1905–06] says "The proteids of serum are electrically inactive. Neither the whole nor any fraction moves in a field. It is not possible to detect a trace of 'ionic' protein. Dialysis or dilution disturbs the equilibrium and 'ionic' globulin appears...". For some reason Hardy's views seem to have been largely neglected. Adair and Robinson [1930, 2], however, say that it is possible that Hardy failed to detect any ionic globulin because of the low ratio of the charge to the mass and the effects of the ion atmosphere. Tiselius [1930] using a considerably more accurate cataphoresis technique than Hardy has examined various artificial mixtures of egg albumin, Bence Jones protein, phycocyan, phycoerythrin and also some native mixtures and concludes that oppositely charged proteins can exist together in solution without influencing each other to any great extent. The concentrations of proteins examined by Tiselius were all less than 0·5 % and it is not possible with his method to examine solutions much stronger than this. The possibility of interaction between proteins at higher concentrations, such as we have been examining in the ultracentrifuge, therefore, still remains and indeed becomes more of a probability in view of Hardy's carefully executed, although somewhat crude, electrophoresis experiments. It is indeed difficult to visualise any other method by which albumin and globulin may influence each other, as they do in the above mixtures, than by virtue of their electric charges, and the fact that the p_H of the medium has, if any, a minor effect does not alter the possibility because the force operative in producing the union would be more a function of the difference between the isoelectric points than of the hydrogen ion concentration of the solution.

By whatever means the two molecules influence each other the important fact which is revealed in the above experiments remains, *viz.* that in some manner the interaction involves a reduction in the molecular size of part of the globulin to approximately the size of albumin. It is impossible to determine on the basis of the available data the real mechanism of this. We may say, however, that the work of Svedberg and collaborators in recent years on the structure of the protein molecule and on the p_H stability region of the proteins makes the idea of a reduction in the size of the globulin molecule, under the conditions with which we are dealing here, not at all such an improbability. When the molecules of both albumin and globulin at p_H values below 4 and above 9 disintegrate in a reversible manner into smaller molecular units [Svedberg and Sjögren, 1930] it is clearly a possibility that in circumstances which may cause an electrochemical union of albumin and globulin the same molecular dimensional forces may be operative and ensure that the resulting product has a molecular weight no greater than 68,000.

There are one or two interesting theoretical aspects of the phenomenon which we have described. If there exists in undiluted serum and concentrated mixtures of albumin and globulin a form of equilibrium between a low molecular fraction and a high molecular fraction, then in the process of centrifugal separation of the two a state of unstable equilibrium must exist. Globulin molecules ought to be continually produced in the solution between the two boundaries, and as these sediment more rapidly than the "albumin" molecules among which they originate, the net result ought to be a steady fall in the area of the "albumin" curve. This has never been found. We do not know, however, what order of velocity constant might be associated with the equilibrium reaction or with the process of aggregation by which the globulin is subsequently formed, and it is conceivable that more time than the centrifuge allows is required for an appreciable formation of globulin in the cell.

If we consider the state of affairs at the "globulin" boundary in undiluted serum it is by no means certain that the concentration of albumin is uniform here. It has been shown that when globulin sediments in a medium containing albumin the sedimentation velocity of the globulin is markedly diminished by the viscous effect of the albumin. It is possible, if not probable, that in the same circumstances the rapidly sedimenting globulin molecules exert a viscous traction on the albumin molecules to cause them to sediment more rapidly. If this should be the case more albumin would cross in unit time a unit cross sectional area in the lower ("globulin"+"albumin") part of the cell than in the middle ("albumin" only) part. The net effect would be again a steady diminution in the area of the "albumin" curve analogous to the effect of the increasing centrifugal force on the protein molecules as they sediment away from the centre of rotation.

As already stated, no such abnormal diminution of the area of the "albumin" curve has been recorded. Without some independent evidence of the existence and extent of such a viscous-attractive force it is, of course, impossible to have any exact idea of the concentration disturbance which it might be expected to give rise to. It is also possible that it might give rise to rather low values for the "globulin" areas by superimposing on the positive globulin concentration gradient a negative "albumin" gradient, but it is not conceivable that it could give rise to effects sufficiently great to furnish an explanation of the phenomenon described here. In addition, any apparent dilution of the "globulin" fraction due to an extraneous cause of this type would also represent a loss in the total protein represented in the sedimentation diagram, and this we know to be constant. Also, no concentration disturbance at the "globulin" boundary can explain the abnormally high concentration and apparent homogeneity of the "albumin" fraction. We can find no explanation for all the facts other than the one advanced here, *viz.* that we are concerned with a truly molecular phenomenon governed by some unknown form of interaction between albumin and globulin.

We have thought it wise to postpone to the end of this paper a consideration of the relationship which the results bear to those of Mutzenbecher, who preceded the writer in this field of work at Upsala. The first experiments which we made with the ultracentrifuge were designed to extend some of Mutzenbecher's experiments, and it was during the course of these experiments that we realised that the phenomenon of a shifting base-line, which was a continual source of trouble to Mutzenbecher, was, at least in part, of mechanical origin. Mutzenbecher believed that it was due to the presence in the solution of low-molecular products, and therefore based all his calculations on the true base-line or x-axis without, at the same time, observing all the technical precautions which Pedersen has shown to be necessary if this is done. Also, whereas the conclusions of the present paper are based on determinations of the individual refraction increment of each fraction in a mixture and depend crucially on the preliminary experimental verification of Lamm's relationships, Mutzenbecher, who had no such experimental verification to rely on, has based his conclusions entirely on calculations of the concentration ratio of the fractions. We believe that these, in the main, are the explanations of the different conclusions arrived at because it is clear that the experimental results in the two cases are in essential agreement.

Mutzenbecher has concluded that there exist in normal horse serum two fractions "identisch mit den rein darstellbaren Fraktionen Albumin und Globulin". We prefer to draw the conclusion that there exist in normal horse serum two fractions of very approximately the same molecular dimensional properties as isolated horse serum albumin and globulin, but considerations of the concentrations in which the two fractions are present suggest strongly that at least the lighter fraction cannot be identical from a chemical structural point of view with pure albumin.

SUMMARY.

1. It has been found possible by means of a modification of Lamm's refractive index method of following protein sedimentation in the ultracentrifuge to obtain satisfactory sedimentation photographs using very concentrated protein solutions.

2. It has been shown that recrystallised horse serum albumin in solution exists in a monodisperse state over a wide range of concentration. Reprecipitated horse serum globulin, on the other hand, has not been obtained in a completely monodisperse form, being always associated to a variable extent with molecules both larger and smaller than itself. It is suggested that much of the disagreement in the literature over the characteristic physico-chemical properties of globulin may be attributed to the variable composition of different preparations of globulin. The composition, from a molecular dimensional point of view, of a particular specimen of globulin remains sensibly the same over a wide range of concentration.

3. A sample of native horse serum, quite untreated in any way, has been examined in the ultracentrifuge and shown to contain two molecular fractions. The lighter fraction represents nearly 80 % of the total protein and the heavier fraction 20 %, despite the fact that by ordinary precipitation analysis the albumin/globulin ratio of the serum is approximately 1.

4. It has been found that on simple dilution of this horse serum the ratio of the two fractions obtained by ultracentrifugal analysis approaches the analytical value for the ratio of albumin to globulin.

5. A series of artificial mixtures of albumin and globulin has been investigated in the ultracentrifuge and it has been shown that only at very low concentrations does the mixture behave like an ideal solution. At all concentrations greater than 0·25 % of total protein the sedimentation constants, boundary spreading coefficients, and concentrations of the two fractions are abnormal. With increasing total concentration there is a steady change in the concentrations of the two fractions so that the lighter fraction always increases in amount and the heavier fraction decreases correspondingly—until the state of affairs present in the original serum is reproduced. The phenomenon is primarily a concentration effect and apparently reversible. The hydrogen ion concentration and nature of the salts in the solution appear to have little, if any, effect.

6. A consideration of the sedimentation rate and degree of spreading at the sedimenting boundaries in this series of mixtures indicates that the lighter species in a concentrated mixture has approximately the same molecular weight as albumin and appears to be homogeneous in the centrifugal field. It is only possible to obtain a very rough idea of the average molecular weight and homogeneity of the heavier species, and the most that the results are able to indicate is that this fraction may be identical with pure globulin.

7. The results indicate that it is not possible to regard the lighter molecular fraction in undiluted horse serum as identical with isolated horse serum albumin.

8. Throughout the work the opportunity has been taken to verify experimentally the basic equations of Lamm's refractive index method.

There will be reported in subsequent papers experiments which employ the new method to investigate the structure of the proteins in normal and pathological sera of various types. Throughout the whole of the work I have been greatly indebted to Prof. Svedberg for his inspiring interest and advice and for the innumerable facilities which he has readily placed at my disposal and which have made the research possible. It gives me the greatest pleasure also to express my gratitude to his assistants, in particular to Dr Pedersen and to Dr Lamm, for much kind help and valuable criticism.

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REFERENCES.

Adair and Robinson (1930, 1). *Biochem. J.* **24**, 993.
—— —— (1930, 2). *Biochem. J.* **24**, 1864.
Gans (1915). *Ann. Physik*, **47**, 709.
Hardy (1905–06). *J. Physiol.* **33**, 251.
Lamm (1928). *Z. physikal. Chem.* **138**, 313.
—— (1929). *Z. physikal. Chem.* **143**, 177.
McFarlane (1932). *Phil. Mag.* **14**, 1.
—— (1933, 1). *J. Sci. Inst.* **10**, 142.
—— (1933, 2). *J. Sci. Inst.* **10**, 208.
Mutzenbecher (1933, 1). *Biochem. Z.* **266**, 226.
—— (1933, 2). *Biochem. Z.* **266**, 250.
—— (1933, 3). *Biochem. Z.* **266**, 259.
Pedersen (1934). *Z. physikal. Chem.* **170**, 41.
Svedberg (1934). *Kolloid Z.* **67**, 1.
—— and Rinde (1924). *J. Amer. Chem. Soc.* **46**, 2677.
—— and Sjögren (1928). *J. Amer. Chem. Soc.* **50**, 3318.
—— —— (1930). *J. Amer. Chem. Soc.* **52**, 2855.
Tiselius (1926). *Z. physikal. Chem.* **124**, 499.
—— (1930). *Nova Acta Reg. Soc. Sci. Upsaliensis*, Ser. 4, **7**, No. 4.
—— (1932). *Kolloid. Z.* **59**, 306.
—— and Gross (1934). *Kolloid Z.* **66**, 11.
Young (1922). *Proc. Roy. Soc. Lond.* B **93**, 15.

XLIX. UNIMOLECULAR FILMS OF LECITHIN AND RELATED COMPOUNDS.

BY ARTHUR HUGHES.[1]

From the Colloid Science Laboratory, Cambridge.

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It is a significant fact that organic molecules such as lecithin, cholesterol and protein, which are known to occur in the membrane of a living cell are all molecules capable of forming unimolecular films at an air-liquid interface. The importance in this connection of the established work on unimolecular films of organic compounds has been realised by Leathes [1923; 1925] and Adam *et al.* [1926; 1928; 1929], who have studied films of lecithin and cholesterol on the surface of water. Their results demonstrated the essential difference between the typical loosely packed expanded film of lecithin and the close-packed condensed film of cholesterol and further indicated the power of cholesterol to bring about a closer packing of lecithin in a mixed film of the two substances.

Gorter and Grendel [1925; 1926] approached the problem from a different angle and showed that the amount of lipoid material in a typical living cell is sufficient to form approximately two molecular layers in the cell membrane.

No precise information has been forthcoming as to the orientation and chemical reactivity of these substances when present in the form of unimolecular films. In view of the fresh light that has been thrown on the physical structure and chemical reactions of molecules by the method of surface potentials [Schulman and Rideal, 1931; Fosbinder and Rideal, 1933], this technique has now been applied in conjunction with force/area measurements, to study the physical and chemical properties of the following compounds: tripalmitin, triolein, lecithin, lysolecithin and cholesterol. A preliminary investigation has also been made of composite films of the above substances with protein.

EXPERIMENTAL.

The surface potentiometer employed in the following work was essentially similar to that previously described by Schulman and Rideal [1931]. In Fig. 1 ΔV, the change in air-liquid potential difference due to the presence of the unimolecular film, is plotted against n, the number of molecules per sq. cm. of surface. The main characteristics of the films are given in Table I and refer to dilute NaCl solution at 17–20°. The two values of n, and the corresponding area per molecule (A), are given for the points where a homogeneous film is first formed on compression and where it finally collapses. The values for the resolved vertical component of the electric moment per molecule (μ) are calculated from the equation $\Delta V = 4\pi n \mu$, and are only relative values.

The limiting areas per molecule show good agreement in those cases previously studied by Adam *et al.* [1926; 1929] by force/area measurements. They find for cholesterol 40·8 sq. Å., for lecithin 114 sq. Å. and for triolein 135 sq. Å. (15°).

Tripalmitin, a typical close-packed condensed film, gives a high moment, due to the glyceride group, of $10{\cdot}0 . 10^{-19}$ E.S.U. which is constant during the small compression range of the film. Triolein, an expanded film with a much greater compression range, gives a slightly higher value of μ owing presumably to the presence of the weakly polar double bonds, but on compression the value approximates to that of tripalmitin. For cholesterol μ remains constant at $4{\cdot}2 . 10^{-19}$ E.S.U. during compression over its small range of about 3 sq. Å. The dipole moment is thus considerably smaller than for lecithin which gives an expanded film of the same type as triolein. μ for lecithin decreases on compression to a much greater extent than for lysolecithin probably owing to the absence of the unsaturated hydrocarbon chain from the latter. In comparing lecithin and lysolecithin the most significant feature is the area of surface occupied per hydro carbon chain, which is 58 sq. Å. for lecithin and 108 sq. Å. for lysolecithin, *i.e.* lysolecithin forms a relatively much more distended film than lecithin. Further, μ for lysolecithin is about half that for lecithin, owing possibly to the removal of one ester linkage.

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[1] Beit Memorial Fellow.

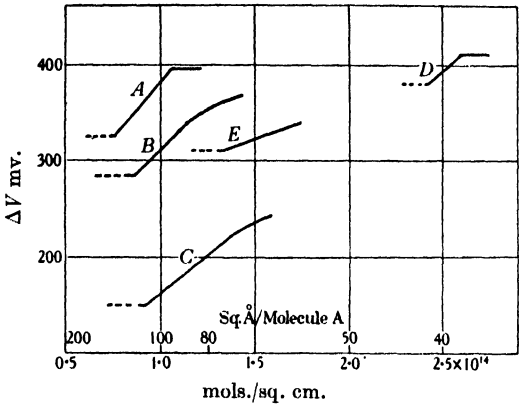

Fig. 1. Surface potentials of unimolecular layers.

A. Triolein NaCl 0·9 %, 19°
B. Lecithin NaCl 0·9 %, 18°
C. Lysolecithin NaCl 0·9 %, 18°
D. Cholesterol NaCl 0·9 %, 17°
E. Lecithin borate p_H 9, 18°

Table I.

Compound	Nature of film	Compression range n	Compression range A sq. Å.	ΔV mv.	μ E.S.U.
Tripalmitin	Solid	$1{\cdot}67 \times 10^{14}$	60·0	636	$10{\cdot}0 \times 10^{-19}$
	Condensed	1·78	56·0	662	9·9
Triolein	Liquid	0·76	132	324	11·3
	Expanded	1·05	95	395	10·0
Cholesterol	Liquid	2·42	41·3	380	4·2
	Condensed	2·60	38·5	410	4·2
Lecithin	Liquid	0·86	116	284	8·8
	Expanded	1·37	73	364	7·1
Lysolecithin	Liquid	0·93	108	150	4·3
	Expanded	1·55	65·5	240	4·1

The molecular weights of lecithin and lysolecithin.

The molecular weights of these two compounds assumed in calculating the area per molecule were the formula weights 805 and 540, corresponding to oleyl-stearyl-lecithin and stearyl-lysolecithin respectively. Price and Lewis [1929] give values for the molecular weight of lecithin by the boiling-point method as 797 in ethyl alcohol and 3388 in benzene. A cold solution of freshly prepared lecithin in alcohol or benzene, or of lysolecithin in chloroform, shows a strong Tyndall cone, which decreases in intensity on boiling. At ordinary temperatures it would appear that both substances are highly polymerised. The rapid spreading of these large particles on an aqueous surface, the ease of compression and extension and the fluid nature of the films suggest that such polymerisation is not due to stable chemical linkages but to a physical process of dipole association between the lecithin or lysolecithin molecules, this union being broken down by the strong attraction of the dipoles to the aqueous surface.

Chemical properties of the films.

(i) ***Effect of*** p_H ***of the underlying solution.*** Films of tripalmitin, triolein and cholesterol are unaltered over the p_H range 2–11. Tripalmitin is hydrolysed slowly and triolein about four times more quickly on *N* NaOH. Fig. 2 shows the effect of p_H on the surface potentials of lecithin and lysolecithin films, using dilute HCl and *M*/25 phthalate, phosphate and borate buffers. The curves for myristic acid and triolein are included for reference. Triolein is a non-ionising molecule and shows no change of surface potential with p_H. Myristic acid, with a single acid ionising group, shows a large fall of surface potential on solutions more alkaline than p_H 3 due to soap formation. With lecithin a fall is again noticed at a p_H higher than 3 due presumably to the ionisation of the phosphoric acid group, which appears to be of the same order of strength as the carboxyl group in myristic acid. The fall is not maintained beyond p_H 6 owing to the amphoteric nature of the molecule. In the case of lysolecithin ionisation begins

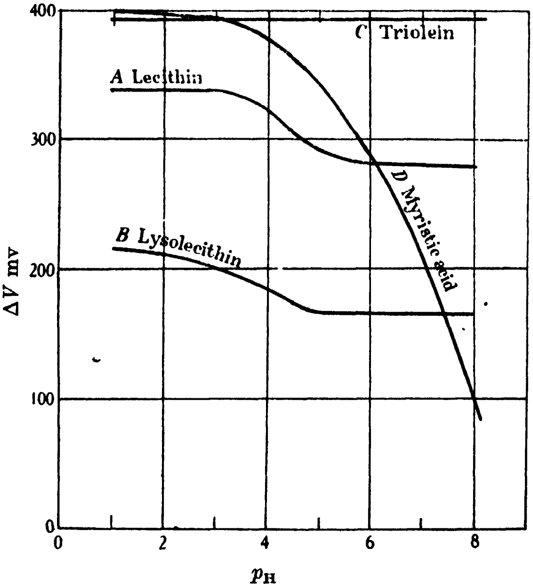

Fig. 2. Effect of p_H on surface potential (ΔV).

A. Lecithin at 100 sq. Å., 20°.
B. Lysolecithin at 100 sq. Å., 20°.
C. Triolein at 95 sq. Å., 20°.
D. Myristic acid at 20 sq. Å., 20°.

at a lower p_H, between 1 and 2, than for lecithin indicating that lysolecithin acts as a stronger acid. An effect peculiar to lecithin films is the change in character on solutions more alkaline than p_H 8, the range of compression becoming much smaller, and implying a much closer packing of the polar head groups (*cf.* Fig. 1).

It is impossible as yet to relate these surface potentials to results of cataphoretic measurements since the latter are not concerned with the total dipole system of the molecule.

(ii) *Oxidation by potassium permanganate.* Fig. 3 shows the change in surface potential with time for films of triolein, lecithin and lysolecithin on 0·1 % $KMnO_4$ in $N/100$ H_2SO_4 at room temperature. Lecithin is attacked rapidly, lysolecithin only very slowly, confirming the absence of an unsaturated hydrocarbon chain from lysolecithin. Triolein is also attacked only very slowly even at the maximum extension of the film to 132 sq. Å. per molecule. The explanation lies in the fact that the area of surface per hydrocarbon chain, 44 sq. Å., is too small to permit the presence of the unsaturated double bonds in the aqueous surface and thus oxidation is inhibited. A similar effect has been observed with oleic acid itself [Hughes and Rideal, 1933].

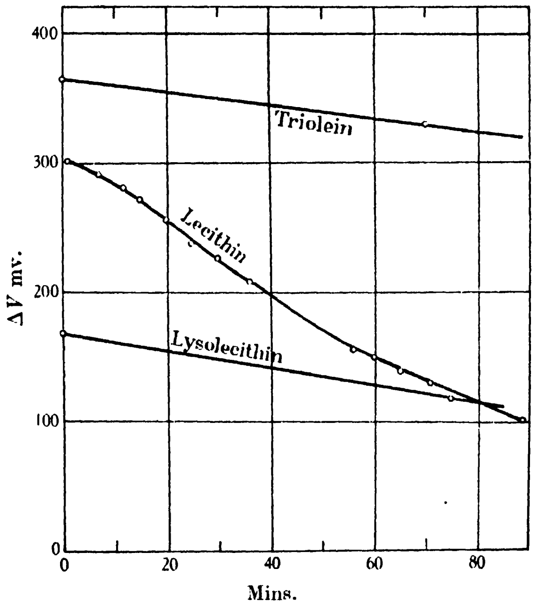

Fig. 3. Rate of oxidation of unimolecular films by 0·1 % $KMnO_4$ in $N/100$ H_2SO_4, 18°.

Mixed films of lecithin and cholesterol.

A series of mixed films of lecithin and cholesterol was examined by the method of surface potentials to ascertain whether any chemical union takes place between the molecules. This would be reflected in a corresponding alteration of the electric moments and hence of the surface potentials. The actual results indicate merely a gradual contracting effect of the cholesterol on the lecithin, except that in the region of equimolecular proportions the film is compressible to values of ΔV higher than the maximum value for lecithin or cholesterol alone, implying a retention of the molecular orientation to higher compressions in these mixtures

(Figs. 4 and 5). Under 20 dynes compression the lecithin in an 80 % cholesterol mixture has an area almost as small as that of cholesterol itself, ca. 40 sq. Å., so that under these conditions the lecithin molecule must be orientated with both hydrocarbon chains nearly vertical, since the minimum area of each chain when vertical is 18·5 sq. Å.

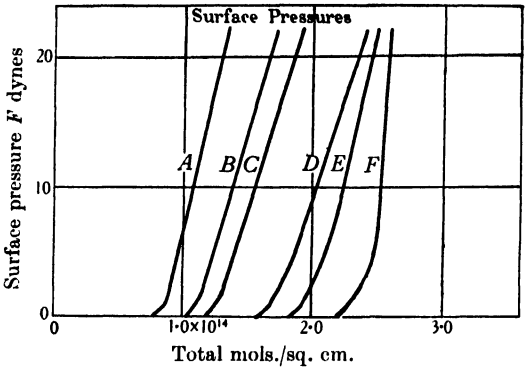

Fig. 4.

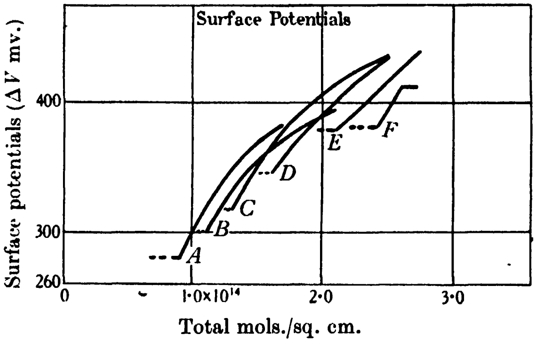

Fig. 5.

Figs. 4 and 5. 15–17°, 0·9 % NaCl. *A*. Lecithin. *B*. 20 % cholesterol. *C*. 40 % cholesterol. *D*. 60 % cholesterol. *E*. 80 % cholesterol. *F*. Cholesterol (% expressed in g. molecules).

Mixed films of protein and fatty molecules.

The possibility of obtaining mixed films of protein and lipoid molecules, and the nature of such films if obtainable, are problems of some importance in connection with current theories of the composition of cell membranes. The work of Mudd and Mudd [1926] on the surface composition of normal and sensitised mammalian blood cells indicates an increased polarity of the cell surface after sensitisation, but there is no information whether the molecules of cholesterol, lecithin and protein can form a composite membrane with properties differing from those of its constituents. With this aim some preliminary experiments have been carried out using a typical protein, egg albumin.

The procedure adopted was first to spread a unimolecular film of the fatty substance on the surface of an aqueous solution and then to inject beneath the film a dilute solution of the protein. It was found that subsequent changes in the physical nature and in the surface potential of the film were affected by the following factors: (i) the p_H of the solution, (ii) the nature of the film-forming molecules, (iii) the surface concentration of the film molecules.

The effect of p_H. On the acid side of the isoelectric point of egg albumin injection of the protein (concentration in trough 0·001 %) causes a rapid rise in the surface potential of a triolein film to a value above that of triolein or of egg albumin. At the same time the liquid film of triolein sets to a rigid gelatinous film similar to that of the protein alone. On the alkaline side of the isoelectric point the rate of gelation of the film becomes progressively slower and is accompanied by a small fall in surface potential. This change, which is complete in 4 minutes at p_H 4·0, takes about 1 hour at p_H 7·0; it cannot be simply a replacement of triolein by protein since the potential of the complex film is always much higher than that of the egg albumin film alone.

Effect of the nature of the film-forming molecule. Films of tripalmitin, hexadecyl alcohol, cholesterol, and lecithin do not appear to be changed by injection of protein at any p_H. Oleic acid behaves in a similar manner to triolein. Lysolecithin is peculiar in that, although gelation of the film takes place very slowly, over a period of hours, an immediate and rapid rise of potential occurs, in 20 minutes at p_H 5. A further study of these complex films is in progress.

SUMMARY.

1. The method of surface potentials has been applied to a study of the physical and chemical properties of unimolecular films of lecithin, lysolecithin, cholesterol, tripalmitin and triolein. The relative electric moments per molecule thus deduced are 8·8, 4·3, 4·2, 10·0, $11{\cdot}3 \times 10^{-19}$ E.S.U. respectively.

2. Lysolecithin forms a liquid expanded film with an upper limiting area of 108 sq. Å., the area per hydrocarbon chain being about twice that for lecithin.

3. Tripalmitin, triolein and cholesterol show no change in properties over a p_H range 2–12. For lecithin a fall in surface potential is noted between p_H 3 and 6, and for lysolecithin between p_H 2 and 5, due to ionisation.

4. On dilute $KMnO_4$ solutions lecithin is oxidised more rapidly than triolein or lysolecithin.

5. Mixed films of protein and fatty molecules have been examined. Homogeneous mixed films are obtainable from egg albumin and triolein.

The lecithin used in these investigations was prepared by Dr Chain by Levene's method through the cadmium chloride compound. It was nearly colourless and gave 3·96 % phosphorus, 1·79 % nitrogen. My sincere thanks are due to Dr Chain for this preparation and to Dr E. J. King for a specimen of pure lysolecithin.

REFERENCES.

Adam and Jessop (1926). *Proc. Roy. Soc. Lond.* A **112,** 362.
—— —— (1928). *Proc. Roy. Soc. Lond.* A **120,** 473.
—— and Rosenheim (1929). *Proc. Roy. Soc. Lond.* A **126,** 25.
Fosbinder and Rideal (1933). *Proc. Roy. Soc. Lond.* A **143,** 61.
Gorter and Grendel (1925). *J. Exp. Med.* **41,** 439.
—— —— (1926). *Proc. K. Akad. Wetensch. Amsterdam,* **29,** 314.
Hughes and Rideal (1933). *Proc. Roy. Soc. Lond.* A **140,** 253.
Leathes (1923). *J. Physiol.* **58,** *Proc.* vi.
—— (1925). *Lancet,* i, 853.
Mudd and Mudd (1926). *J. Exp. Med.* **43,** 127.
Price and Lewis (1929). *Biochem. J.* **23,** 1030.
Schulman and Rideal (1931). *Proc. Roy. Soc. Lond.* A **130,** 259.

L. THE ACTION OF SNAKE VENOMS ON SURFACE FILMS.

BY ARTHUR HUGHES.[1]

From the Colloid Science Laboratory, Cambridge.

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THE work of Kyes [1902] and of Kyes and Sachs [1903] showed that the action of snake venom haemolysins is upon the lecithin portion of the cell membrane. Later, Delezenne and Fourneau [1914] found that egg-lecithin undergoes a partial hydrolysis by snake venom, the unsaturated fatty acid residues being specifically removed to form lysolecithin, which contains a single saturated aliphatic hydrocarbon chain. No satisfactory mechanism has been suggested for this specific hydrolysis. A study has been made in the present work of the actions of various snake venoms on unimolecular films of lecithin and other compounds in relation to the processes involved in venom haemolysis.

As opposed to the study of reactions in bulk, the technique of surface films has the advantage that the enzyme substrate can be studied in a definite and reproducible state, that of an oriented monolayer. Chemical reactions occurring therein are observable by the accompanying changes in the surface potential of the film. The surface potentiometer employed has been described elsewhere [Schulman and Rideal, 1931].

Preliminary investigation.

The following venoms were available:

COLUBRIDAE:	Black snake	*Pseudechis porphyriacus.*
	Copperhead	*Denisonia superba.*
	Black tiger	*Notechis scutatus.*
	Cobra	*Naia naia.*
VIPERIDAE:	Daboia	*Vipera elegans.*

A film of lecithin was spread on the surface of dilute NaCl solution in the trough of the surface potentiometer and compressed to a fixed reference area per molecule, usually 100 sq. Å. A dilute solution of venom was then injected under the film from the far side of the movable glass barrier enclosing the film. The solution was well mixed and the change of surface potential was measured. In cases where the change was rapid it was found advisable to mix the venom solution in the trough prior to spreading the film to ensure uniformity of the solution. The lecithin film is spread immediately after cleaning the surface, since protein, which is always present with the venom, accumulates at the free liquid surface in the more concentrated solutions.

With a venom concentration of 0·01 % in the underlying solution a rapid fall in surface potential takes place, *i.e.* in the direction of a conversion of lecithin into lysolecithin (*cf.* Fig. 1), in the case of all the venoms except cobra. This anomaly of cobra venom, ascribable to the different nature and greater proportion of associated protein, will be referred to later. The final values of the surface potential of a lecithin film on the other four venoms depend on the degree to which the associated protein is adsorbed under a lysolecithin film. Thus injection of venom under a lysolecithin film causes a small rise in potential to a value within about 15 mv. of the final value of lecithin on that solution.

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[1] Beit Memorial Fellow.

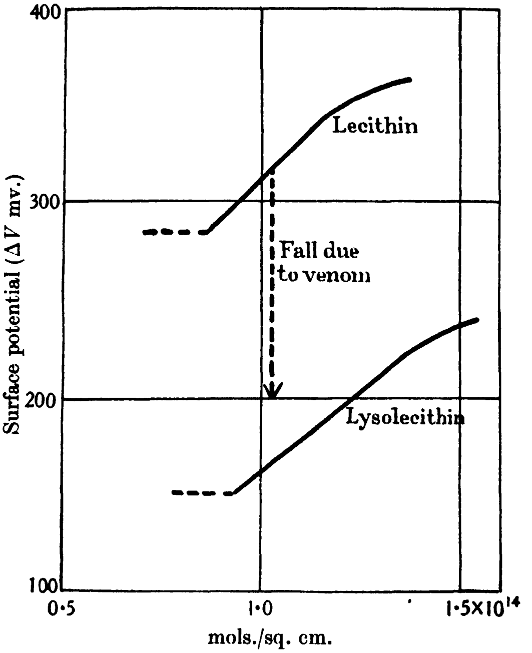

Fig. 1. Surface potentials of lecithin and lysolecithin, 0·9 % NaCl, 18°.

None of the venoms has any effect on a unimolecular film of cholesterol or of protein, and they cause no hydrolysis of tripalmitin, triolein, cerebron or sphingomyelin.

The action of the venom on a lecithin film was found to depend on three main factors:

(i) The p_H of the underlying solution.
(ii) The surface concentration of the lecithin film.
(iii) The venom concentration.

These effects were examined in detail.

(i) *The effect of* p_H. The action of black tiger venom was studied over a p_H range from 4 to 11 using $M/25$ phthalate, phosphate and borate buffers and a fixed venom concentration of 0·001 %. Fig. 2 gives a family of curves showing the change of surface potential (ΔV) with time at various p_H values for a definite initial surface concentration of lecithin ($n = 1{\cdot}0 . 10^{14}$ mols./sq. cm.). There is no reaction on solutions more acid than p_H 4·8, the velocity at p_H 6·0 is about equal to that at p_H 8·0, while at p_H 10·8 the reaction is again strongly inhibited. The optimum is at about p_H 7·3.

The venom itself is stable in fairly strongly acid media but not in alkaline. After keeping for 3 hours in $N/10$ HCl at room temperature and returning to p_H 7·2 the venom solution shows unimpaired activity. Treatment with $N/10$ NaOH for the same period destroys the venom almost completely as regards its action on a lecithin film. The action is not inhibited in the presence of 0·5 % NaF.

(ii) *Effect of surface concentrations of lecithin.* The rate of attack of a lecithin film is considerably diminished as the number of molecules of lecithin per sq. cm. is increased (Fig. 3). The time for complete hydrolysis of the film increases from 7 to 100 minutes for a doubling of the initial surface concentration of lecithin, from 1·04 to 2·07. 10^{14} mols./sq. cm. It is known that the venom specifically removes the unsaturated hydrocarbon chain from lecithin. A triolein film is not hydrolysed even very slowly by venom, the action of which cannot therefore be concerned only with a coupling with the unsaturated group in lecithin. It must also couple with some other point in the lecithin molecule. Compression of the lecithin will alter the spacing of the essential points of attachment, and at the higher compressions the double bonds will be removed from the aqueous surface, as observed in the case of oleic acid [Hughes and Rideal, 1933]. It may thus be suggested that the lecithinase embodies also a spacing of two active groups

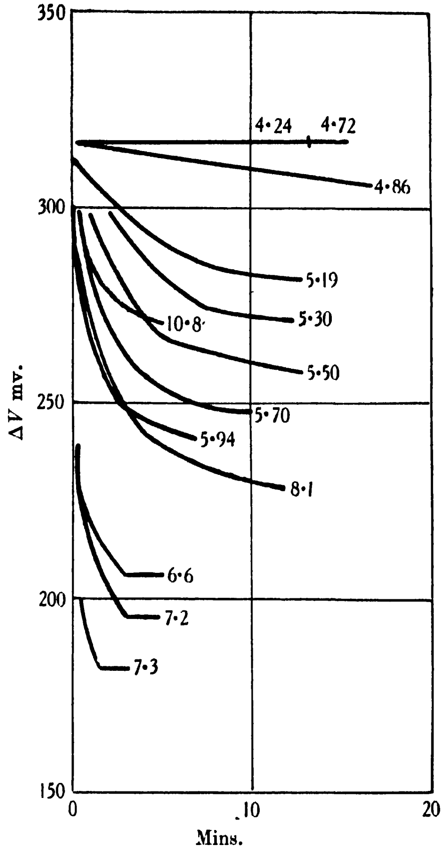

Fig. 2.

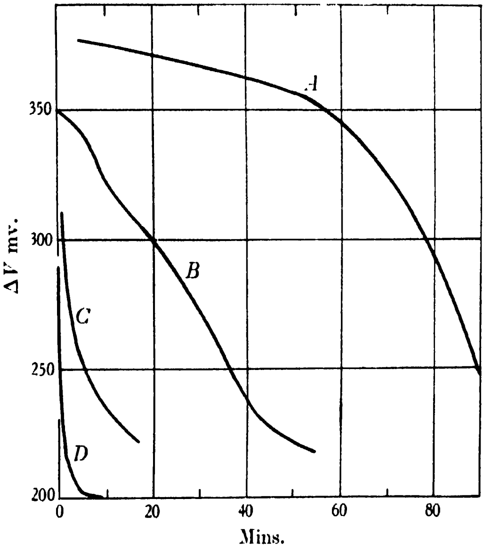

Fig. 3.

Fig. 2. Influence of p_H on attack of lecithin films by black tiger snake venom, 20°.

Fig. 3. Effect of compression of lecithin film on attack by black tiger venom (0·001 %).

$$\left.\begin{array}{ll} A & n=2{\cdot}11.10^{14}\text{ mols./sq. cm.}\\ B & n=1{\cdot}57.10^{14}\text{ mols./sq. cm.}\\ C & n=1{\cdot}27.10^{14}\text{ mols./sq. cm.}\\ D & n=1{\cdot}04.10^{14}\text{ mols./sq. cm.}\end{array}\right\} p_H\ 7{\cdot}2,\ 17^\circ$$

which coincide with a similar spacing of two active groups in the distended lecithin molecule for the maximum probability of reaction.

(iii) *Effect of venom concentration.* Fig. 4 shows the rate of attack of a lecithin film from a fixed initial surface potential of 280 mv. by varying concentrations of copperhead venom, in $M/30$ phosphate buffer at p_H 7·2.

It is seen that at concentrations higher than about 10^{-4} g. venom 100 ml. (1 part in a million), the reaction is of zero order and complete in 5 minutes at 20°. Below this concentration and down to the lowest concentration used, 1 part in 40 millions, the rate of reaction falls off sharply with the venom concentration till at $2{\cdot}5 . 10^{-6}$ % the half life is about 1 hour. It is impossible to give quantitative values for the velocity constants at these low concentrations owing to the varying amounts of protein in the solution and the possible adsorption of the venom on the glass vessel as well as at the surface of the film.

It must be remembered that the values of the venom concentration refer to dry weight of crude venom scale, and that the fraction of this which is pure active principle is unknown.

Attack of lecithin/cholesterol films by venom.

The rate of attack by black tiger venom of a film containing 20 % cholesterol molecules is the same as that for lecithin alone at the same area per molecule. In a 50 % mixture the velocity is not appreciably diminished, but in an 80 % cholesterol film no reaction is observed over a period of hours. It appears that cholesterol has no specific inhibitory power in this reaction other than that due to its causing a general contraction of the lecithin film as the proportion of lecithin is decreased. As shown in Fig. 3, a similar inhibition is observed by mere compression of a pure lecithin film.

Correlation of haemolytic action of venoms with their action on lecithin films.

The following experiments were carried out to ascertain how far the observed changes brought about in a lecithin film by venom were ascribable to the haemolysin of the venom.

Washed guinea-pig cells were used throughout. In Table I a comparison is shown of the effect of black snake venom in haemolysis and on the surface potential of a lecithin film. The venom solution was used (*a*) unboiled, (*b*) boiled 1 hour in saline, (*c*) unboiled but filtered though a Seitz filter.

Table I.

1 ml. 5 % cells, 1 ml. venom solution (1 mg./ml. in saline) at 37°.

	Unboiled unfiltered	Boiled 1 hr. unfiltered	Unboiled filtered
Haemolysis	C.H. 20 mins.	C.H. 20 mins.	N.H. 20 mins. C.H. 40 mins.
Film activity: fall of ΔV in 10 mins.	46 mv.	41 mv.	22 mv.

C.H. = Complete haemolysis. N.H. = No haemolysis.

These figures demonstrate a definite relationship between hydrolysis of a lecithin film and haemolysis, by black snake venom, and further show the remarkable stability of the venom to prolonged boiling in saline, a point which is discussed more fully later.

A more complete set of experiments was carried out using four varieties of venom: copperhead, daboia, cobra and black tiger, while a definite p_H was maintained with isotonic phosphate p_H 7·4. Control experiments on the isotonic buffers employed showed that these had no effect on the fragility of the cells. The system for haemolysis was made up as follows: 1 ml. of venom solution in isotonic saline (1 mg. per ml.), 1 ml. isotonic phosphate buffer and 0·5 ml. of 5 % washed guinea-pig erythrocytes. The venom solution was used (*a*) unboiled, (*b*) boiled 10 minutes, (*c*) boiled 60 minutes, the heating being carried out at p_H 7·4. It was immediately noticed that the cobra venom solution on heating behaved very differently from the other venoms in giving a bulky yellowish white precipitate, whereas the other solutions only showed a slight opalescence even after 1 hour's boiling. This and other anomalies of cobra will be discussed later.

The main results were, in the first place, that ability to hydrolyse a lecithin film does not necessarily imply haemolytic power on red cells of any given species. Thus daboia gave no haemolysis, black tiger only a trace after 16 hours, while copperhead and cobra as well as black snake were strongly haemolytic, showing complete haemolysis in 2 hours with a concentration of 0·2 mg./ml.

Secondly, after 10 minutes' boiling at p_H 7·4 no haemolysis was detectable with any of the solutions. This result in comparison with the results shown in Table I indicates that the heat stability of the venom depends on the p_H of the solution. This question was investigated more closely. It was found for copperhead and cobra that 15 minutes' boiling at p_H 5·9 (phosphate) has no effect on copperhead and only a slight effect on cobra, while apparently complete destruction ensues at p_H 7·0 (phosphate) and at p_H 9·0 (borate) (Table II).

Table II.

1 drop 50 % cells, 1 ml. venom solution (1 mg./ml. in isotonic buffer) incubated at 37°. for 2 hrs. then at room temperature.

p_H	5·9 phosphate	7·0 phosphate	9·0 borate
Cobra			
Unboiled	C.H. 3–3½ hrs.	C.H. 3–3½ hrs.	C.H. 1¾ hrs.
Boiled 15 mins.	P.H. 3–3½ hrs.	N.H.	N.H.
Copperhead			
Unboiled	C.H. 1¾ hrs.	C.H. 1¾ hrs.	P.H. 3–3½ hrs.
Boiled 15 mins.	C.H. 1¾ hrs.	N.H.	N.H.

C.H. = Complete haemolysis. P.H. = Partial haemolysis. N.H. = No haemolysis in 20 hrs.

This sharp effect of p_H on heat stability has not previously been examined and may account for varied reports as to the influence of heat on different venoms [*cf.* Phisalix, 1922].

The experiments with surface films of lecithin revealed further that where complete destruction of the venom is registered with regard to haemolysis, an appreciable quantity of lecithinase may still be present. It has been already mentioned that a concentration of venom as low as $2{\cdot}5.10^{-6}$ % is detectable by means of surface potentials, while a concentration of $1{\cdot}5.10^{-3}$ % venom only produces complete haemolysis in 16 hours (*cf.* Table III and Fig. 4).

Table III. *Effect of venom concentration on haemolysis by copperhead.*

Conditions as in Table II, p_H 7·0 phosphate.

% venom ...	10^{-1}	5.10^{-2}	$2{\cdot}5.10^{-2}$	$1{\cdot}2.10^{-2}$	$6{\cdot}2.10^{-3}$	$3{\cdot}1.10^{-3}$	$1{\cdot}5.10^{-3}$	$7{\cdot}5.10^{-4}$
Time 1¾ hrs.	4	3	3	3	3	1	–	–
3½ hrs.	4	4	4	4	4	4	1	–
16 hrs.	4	4	4	4	4	4	4	3

(4 represents complete haemolysis.)

It is seen that at a concentration of $7{\cdot}5.10^{-4}$ % venom haemolysis is not yet complete even in 16 hours, whereas a lecithin film is completely transformed in 3 minutes by the same venom concentration. The method of surface potentials provides therefore a very much more sensitive check on the presence of a lecithinase haemolysin.

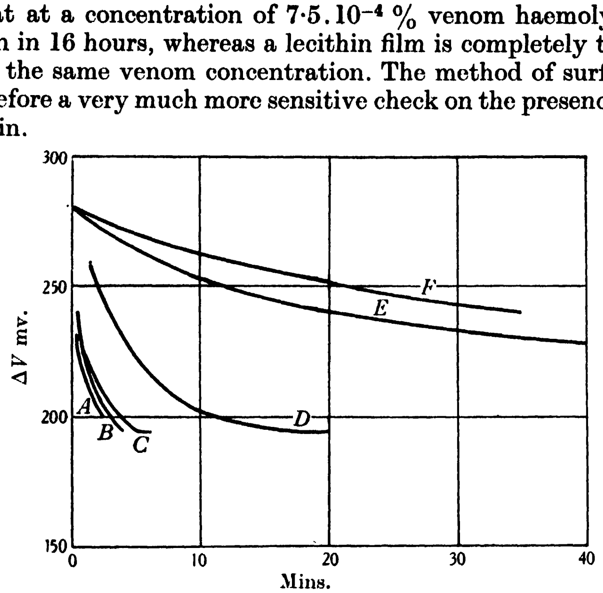

Fig. 4. Attack of lecithin films by copperhead venom, p_H 7·2, 20°.

Venom concentrations: A 5×10^{-4}. B 1×10^{-4}. C 5×10^{-5}. D 1×10^{-5}. E 5×10^{-6}. F $2{\cdot}5\times10^{-6}$. g. %.

The surface activities of venom solutions boiled at various p_H values were compared with the activity of the same venom on progressive dilution and the results are summarised in Table IV. The quantity of undecomposed venom is estimated from the rate of attack on a lecithin film.

Table IV.

Effect of boiling on the lecithinase content of copperhead venom. Initial weight of venom 10^{-3} g.

p_H ...	5·9	6·5	7·2	9·0
Boiled for 15 mins.	10^{-3}	10^{-3}	*ca.* 10^{-5}	nil g. venom remaining
Boiled for 60 mins.	10^{-3}	*ca.* 10^{-4}	*ca.* 10^{-6}	,, ,, ,,

The active principle of the venom is therefore rapidly decomposed on the alkaline side of a sharp p_H limit of 6·5–7·0 and boiling for 15 minutes at p_H 7·2 will inactivate the venom as far as haemolytic experiments can decide, by reducing the venom concentration a hundredfold, although this small quantity, 10^{-5} g., is still readily detected by the method of surface potentials. The complete loss of surface activity as well as haemolytic activity on boiling at p_H 9·0 seems convincing evidence of the close relation of the two effects.

An attempt was made to examine the influence of p_H on the rate of venom haemolysis. It is probably impossible in some cases to separate the effect of p_H from that of the buffering ion. For cobra and copperhead venoms the rate of attack was about the same in two isotonic phosphate buffers at p_H 5·9 and 7·0, but whereas cobra is considerably more active at p_H 9·0 (borate), copperhead is considerably less so (*cf.* Table II). This effect of p_H on copperhead haemolysis supports the results of Holden [1934] that rabbit cells are haemolysed more rapidly at p_H 5·6 than at p_H 8·0.

The anomalies of cobra venom.

Three different specimens of cobra venom were examined, and the behaviour was found to be uniformly different from that of the other venoms studied. At a concentration of 0·01 % and down to 0·0001 % cobra venom gives no detectable hydrolysis of a lecithin film, but on diluting to 0·00005 % (1 part in 2.10^6) a slow reaction takes place as in the case of the other venoms. The inactivity of cobra venom in higher concentration is possibly related to the protective action of excess of this particular venom on the haemolysis of erythrocytes [Kellaway and Williams, 1933]. This inhibition occurs to a negligible extent with the Australian venoms. It is possible that there is a marked preferential adsorption of the protein portion of cobra venom on a lecithin film, and at a concentration sufficient to form a complete layer of protein at the surface the lecithinase is prevented from reaching the lecithin. It is indeed found that the presence of such a quantity of cobra venom will almost completely inhibit the attack of a lecithin film by other normally active venoms such as black tiger or daboia. Again, addition of an equal weight of egg albumin to black tiger venom reduces its rate of attack on a lecithin film tenfold.

Another anomaly of cobra venom has recently been stressed by McFarlane and Barnett [1934], namely its anticoagulant action on plasma, as opposed to the strong coagulant action of Russell's viper (daboia) venom and certain other venoms. It is significant that attempts to separate the toxins from the coagulant principle in these cases have so far failed. There is thus a possibility of a close relation between the coagulant and the lecithinase.

Pre-haemolytic swelling.

In cases of venom haemolysis a large pre-haemolytic swelling of the erythrocytes is usually observed. If the first stage of haemolysis is indeed the liberation of lysolecithin in the lipoidal surface layer of the cell membrane one would anticipate a considerable increase in area since the area per hydrocarbon chain in lysolecithin films is nearly double that obtaining in a lecithin film at the same state of compression. The more expanded nature of the film thus formed would be accompanied by an increased fragility and permeability.

SUMMARY.

1. The physico-chemical properties of snake venom have been examined through its reaction with unimolecular films, particularly lecithin films. Five varieties of venom were studied: black snake, black tiger, copperhead, daboia and cobra.

2. The rate of hydrolysis of a unimolecular film of lecithin to lysolecithin by venom lecithinase is dependent on p_H, surface concentration of lecithin molecules and the venom concentration. The p_H optimum is about 7·3. Compression of the lecithin molecules greatly decreases the rate of hydrolysis. Venom concentrations as low as 1 part in 40 millions are detected by the method of surface potentials.

3. The lecithinase is stable to prolonged boiling at p_H 5·9, but is rapidly destroyed on boiling in solutions more alkaline than p_H 7·0.

4. Haemolysis conducted concurrently with experiments on surface films shows a direct relation between haemolysis and lecithinase content as measured by rate of attack on a lecithin film.

5. Anomalies of cobra venom are discussed.

I wish to acknowledge my indebtedness to Mr E. T. C. Spooner for his invaluable collaboration in the experiments on haemolysis, and further to Dr C. H. Kellaway, whose gifts of snake venom made the work possible. My thanks are also due to Prof. E. K. Rideal for his constant interest and much helpful criticism. I am indebted to Dr Chain for fresh specimens of egg-lecithin used in the above experiments, prepared by Levene's method. The analysis figures were 3·96 % phosphorus, 1·79 % nitrogen.

REFERENCES.

Delezenne and Fourneau (1914). *Bull. Soc. Chim.* **15**, 421.
Holden (1934). *Austral. J. Exp. Biol. Med. Sci.* **12**, 55.
Hughes and Rideal (1933). *Proc. Roy. Soc. Lond.* A **140**, 253.
Kellaway and Williams (1933). *Austral. J. Exp. Biol. Med. Sci.* **11**, 84.
Kyes (1902). *Klin. Woch. Berlin*, **39**, 889, 918.
—— and Sachs (1903). *Klin. Woch. Berlin*, **40**, 21, 57.
McFarlane and Barnett (1934). *Lancet* (ii), 985.
Phisalix (1922). Les animaux venimeux et les venins, **2**, 478.
Schulman and Rideal (1931). *Proc. Roy. Soc. Lond.* A **130**, 259.

LI. VARIATIONS IN SERUM-MAGNESIUM AND IN THE PARTITION OF SERUM-CALCIUM IN NORMAL PARTURITION AND IN MILK FEVER.

By WILLIAM GODDEN and JOHN DUCKWORTH.

(*From the Rowett Research Institute, Aberdeen.*)

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The studies presented by Fish [1930] showed that milk fever usually occurred with a serum-Ca average level of 4·1 mg. per 100 ml. of serum, whilst the serum-inorganic P level averaged 2·43 mg. His work confirmed the results of Little and Wright [1925]. Since the work of Fish an increasing amount of attention has been given to the partition of calcium in different pathological conditions including milk fever. Sjollema and Seekles [1930] have reported an average Ca ion value of 0·44 mg. per 100 ml. in the serum of milk fever cows as compared with an average normal value of 1·65 mg. Little and Mattick [1933] reported that the diffusible serum-Ca was never above 3 mg. per 100 ml. in milk fever as compared with the normal value of 5 mg.

Benjamin and Hess [1933] demonstrated that serum-Ca may be divided into four fractions: (1) the Ca ion, (2) an ultrafiltrable Ca complex, adsorbable on barium sulphate, (3) a non-ultrafiltrable Ca complex, adsorbable on barium sulphate, and (4) the so-called "protein-bound" Ca. It was thought that additional information on the changes in blood composition at the time of parturition or during milk fever might be obtained by the application of the technique of Benjamin and Hess. Owing to the fact however that cases of milk fever occurred before a complete study of the most suitable method of ultrafiltration had been made it was possible to make a division into two fractions only, namely the total adsorbable calcium (2+3 of Benjamin and Hess) and protein-bound calcium *plus* Ca ion (1+4 of Benjamin and Hess).

A study of the literature reveals very little definite information as to the changes in serum-Mg consequent upon milk fever. Sjollema and Seekles [1930] give the average value for serum-Mg in normal cows as 1·66 mg. per 100 ml. and in cases of milk fever 2·19 mg. per 100 ml. This latter figure is the average for a number of cows from which the blood sample was taken when the symptoms were acute. As will be shown later, this does not appear to be the time when the magnesium level is at its highest.

EXPERIMENTAL.

For several years a group of non-pedigree Ayrshire cows had been under observation and records of the variation in their blood composition were available as a result of regular sampling at 3-weekly intervals. At the period of the 1934 calvings, it was decided to obtain frequent samples of blood from each cow during the few days immediately before and after calving, one sample being drawn, whenever possible, immediately after parturition. The cows varied in age from 8 to 10 years and as it happened this year there were three typical cases of milk fever, three abnormal cases showing some signs of milk fever, but having other complications, and three normal calvings. We were thus able to make a comparison of the changes occurring in the composition of the blood serum under these three conditions.

Analytical methods.

(1) *Serum-magnesium.* The precipitation method of Denis [1922] was modified as follows. Into a 15 ml. conical centrifuge-tube were measured 1 ml. of concentrated ammonia (sp. gr. 0·880) and then 1 ml. of 5 % ammonium phosphate followed by 3 ml. of the supernatant liquid from the calcium estimation. The precipitate formed when the first two solutions were mixed entirely dissolved on adding the third and then the magnesium ammonium phosphate separated out in a coarsely crystalline form. The precipitate was separated and washed centrifugally, using Holzapfel's washing method [1934], and was then dissolved and the blue colour developed by the method of Fiske and Subbarow [1925], as modified by Stewart [1933], and matched in the usual manner.

(2) *Serum-calcium.* The method of Clark and Collip [1925] was used.

(3) *Adsorbable calcium complexes.* The calcium adsorbable on barium sulphate was determined by the method of Benjamin and Hess [1933]. The difference between this and total serum-calcium represented the "protein-bound" calcium *plus* ionised calcium.

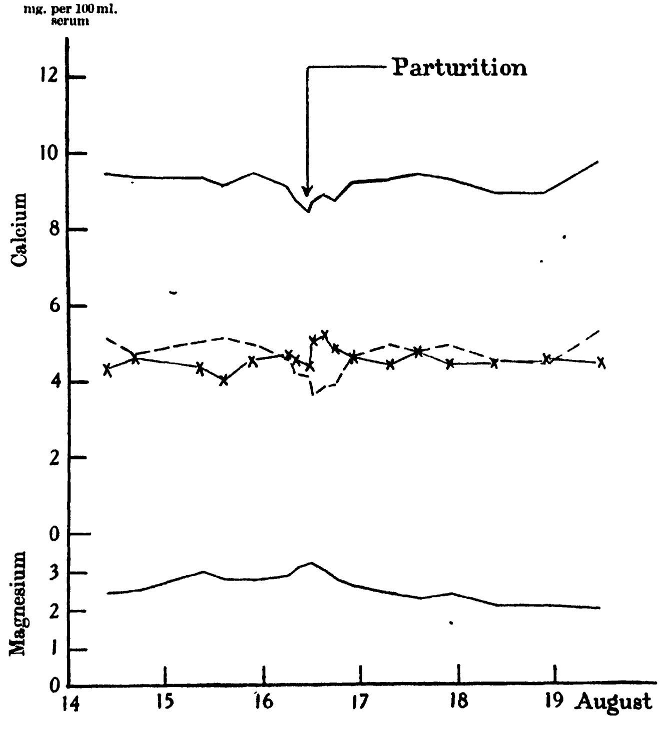

Fig. 1. Cow no. 13. Normal case.

—— Total serum-Ca; x — x non-adsorbable Ca; — — — adsorbable Ca; lower ———— serum-Mg.

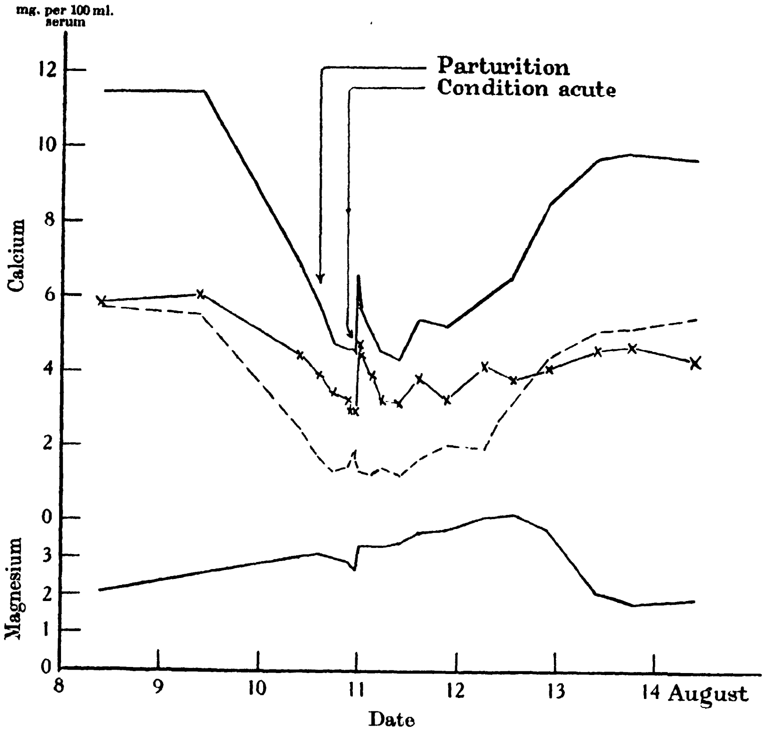

Fig. 2. Cow no. 18. Milk fever case. Description as fig. 1.

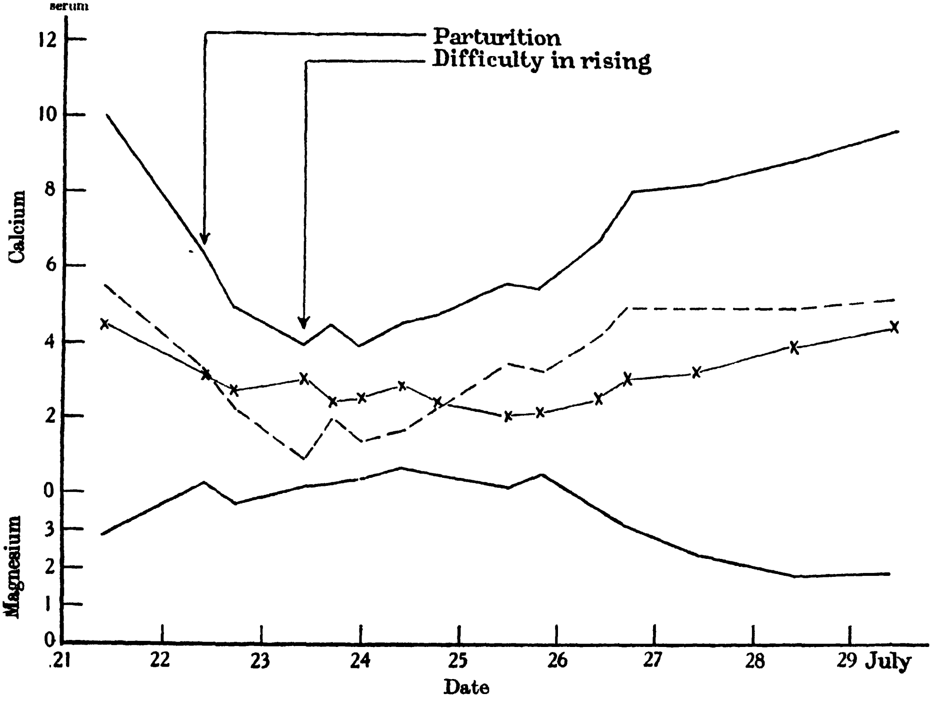

Fig. 3. Cow no. 12. Doubtful case. Description as fig. 1.

Samples of blood were, in all cases, drawn from the jugular vein, employing partial stasis. The serum was separated from the blood clot within 18 hours of drawing the blood.

Typical results illustrating the changes in these four constituents of the serum during normal calvings, milk fever cases and the three doubtful cases are shown in Figs. 1, 2 and 3 respectively. The detailed data for each of the 9 cows are given in Table I and this is followed by the individual case histories. In all cases where calcium gluconate was injected, a 10 % solution (containing 0·91 g. Ca per 100 ml.) was used.

Table I. *Composition of the blood serum of the experimental cows.*

All values in mg. per 100 ml.

Date	Time	Serum-Mg	Total serum-Ca	Adsorbable Ca complexes	"Protein-bound" Ca and Ca ion
Cow no. 13. Normal calving (see Fig. 1).					
Aug. 14	10.00 a.m.	2·42	9·4	5·1	4·3
	5.00 p.m.	2·46	9·3	4·7	4·6
„ 15	9.00 a.m.	3·00	9·3	5·0	4·8
	3.00 p.m.	2·78	9·1	5·1	4·0
	10.00 p.m.	2·81	9·4	4·9	4·5
„ 16	6.00 a.m.	2·90	9·1	4·5	4·6
	9.00 a.m.	3·12	8·7	4·2	4·5
	11.30 a.m.*	3·15	8·4	4·1	4·3
	12.30 p.m.	3·12	8·7	3·7	5·0
	3.15 p.m.	2·98	8·9	3·8	5·1
	6.15 p.m.	2·78	8·7	3·9	4·8
	10.00 p.m.	2·63	9·2	4·6	4·6
„ 17	6.45 a.m.	2·41	9·3	4·9	4·4
	2.30 p.m.	2·31	9·4	4·7	4·7
	10.00 p.m.	2·41	9·3	4·9	4·4
„ 18	10.00 a.m.	2·11	8·9	4·5	4·4
	10.00 p.m.	2·11	8·9	4·4	4·5
„ 19	11.00 a.m.	2·04	9·7	5·3	4·4
Cow no. 5. Normal calving.					
Aug. 1	10.00 a.m.	2·86	9·7	5·6	4·1
„ 2	10.00 a.m.	2·74	9·4	4·4	5·0
„ 3	9.30 a.m.	2·74	8·2	4·8	3·4
	12.10 p.m.	2·60	7·5	4·1	3·4
	12.55 p.m.*	2·50	7·5	4·5	3·0
	5.00 p.m.	2·68	7·2	3·6	3·6
	10.00 p.m.	2·40	7·5	3·5	4·0
„ 4	10.00 a.m.	2·02	8·0	4·2	3·8
	7.00 p.m.	2·17	8·3	4·3	4·0
„ 5	11.00 a.m.	2·60	7·8	3·6	4·2
„ 6	10.00 a.m.	2·60	8·3	4·5	3·8
	5.00 p.m.	2·52	9·9	5·5	4·4
Cow no. 9. Normal calving.					
Oct. 15	7.30 a.m.	3·00	9·6	4·3	5·3
	10.15 a.m.	3·23	9·1	4·1	5·0
	2.30 p.m.*	3·33	8·7	4·3	4·4
	10.00 p.m.	3·49	8·9	4·5	4·4
„ 16	9.30 a.m.	2·61	8·8	5·2	3·6
„ 17	9.30 a.m.	2·86	9·0	5·3	3·7
„ 20	9.30 a.m.	2·80	9·3	4·0	5·3

* An asterisk indicates time of calving.

Table I (*cont.*).

Date	Time	Serum-Mg	Total serum-Ca	Adsorbable Ca complexes	"Protein-bound" Ca and Ca ion
Cow no. 18. Milk fever case (see Fig. 2).					
Aug. 8	10.00 a.m.	2·10	11·5	5·7	5·8
„ 9	10.00 a.m.	2·50	11·5	5·5	6·0
„ 10	10.00 a.m.	3·04	6·8	2·4	4·4
	2.00 p.m.*	3·12	5·7	1·8	3·9
	5.30 p.m.	3·04	4·7	1·3	3·4
	8.50 p.m.	2·92	4·6	1·4	3·2
	9.55 p.m.	2·81	4·6	1·7	2·9
	10.45 p.m.	2·71	4·5	1·6	2·9
	11.15 p.m.	2·80	6·6	1·9	4·7
„ 11	12.15 a.m.	3·26	5·7	1·3	4·4
	3.00 a.m.	3·26	5·0	1·2	3·8
	5.30 a.m.	3·34	4·6	1·4	3·2
	10.00 a.m.	3·36	4·3	1·2	3·1
	2.30 p.m.	3·65	5·4	1·6	3·8
	9.00 p.m.	3·75	5·2	2·0	3·2
„ 12	6·30 a.m.	4·11	6·0	1·9	4·1
	12.15 p.m.	4·22	6·5	2·7	3·8
	9.30 p.m.	3·80	8·5	4·4	4·1
„ 13	10.00 a.m.	2·14	9·7	5·1	4·6
	5.00 p.m.	1·85	9·9	5·2	4·7
„ 14	10.00 a.m.	1·91	9·8	5·5	4·3
Cow no. 7. Milk fever case.					
July 11	9.30 a.m.	2·42	10·3	5·1	5·2
	4.30 p.m.	2·52	9·7	4·6	5·1
„ 12	12.15 a.m.	2·88	8·9	3·6	5·3
	9.30 a.m.	2·98	6·9	2·1	4·8
	2.00 p.m.*	3·30	6·0	2·9	3·1
	5.30 p.m.	3·22	5·5	2·8	2·7
	10.00 p.m.	3·00	5·1	2·5	2·6
„ 13	2.30 p.m.	3·09	3·9	0·5	3·4
	10.30 p.m.	3·12	3·8	0·6	3·2
„ 14	9.30 a.m.	3.66	6·0	2·3	3·7
	5.00 p.m.	3·12	6·7	2·9	3·8
„ 15	10.00 a.m.	2·34	9·7	4·4	5·3
Cow no. 15. Milk fever case.					
July 17	9.30 a.m.	2·91	10·3	5·2	5·1
	5.00 p.m.	2·94	10·5	5·6	4·9
„ 18	9.30 a.m.	2·70	10·3	4·7	5·6
	8.30 p.m.	2·88	10·4	5·8	4·6
„ 19	10.00 a.m.	3·33	6·4	2·7	3·7
	2.00 p.m.*	3·26	5·0	1·6	3·4
	7.45 p.m.	3·61	3·8	0·8	3·0
	10.15 p.m.	4·00	3·8	1·1	2·7
„ 20	5.30 a.m.	4·11	3·2	1·3	1·9
	9.45 a.m.	4·69	4·6	1·6	3·0
	4.45 p.m.	4·35	4·1	1·4	2·7
	10.00 p.m.	4·69	4·4	1·3	3·1
„ 21	10.00 a.m.	4·17	4·9	2·5	2·4
	5.00 p.m.	4·35	5·4	2·7	2·7
„ 22	10.00 a.m.	3·38	6·4	2·6	3·8
„ 23	5.00 p.m.	1·41	11·1	6·0	5·1
„ 25	10.00 a.m.	2·03	9·7	4·7	5·0

* An asterisk indicates time of calving.

Table I (*cont.*).

Date	Time	Serum-Mg	Total serum-Ca	Adsorbable Ca complexes	"Protein bound" Ca and Ca ion
Cow no. 12. Doubtful case (see Fig. 3).					
July 21	10.00 a.m.	2·88	10·0	5·5	4·5
,, 22	9.15 a.m.*	4·30	6·5	3·3	3·2
	5.00 p.m.	3·82	5·0	2·2	2·8
,, 23	10.00 a.m.	4·17	4·0	0·9	3·1
	5.00 p.m.	4·27	4·5	2·0	2·5
	10.15 p.m.	4·35	4·0	1·4	2·6
,, 24	10·00 a.m.	4·72	4·6	1·7	2·9
	5.00 p.m.	4·50	4·8	2·3	2·5
,, 25	10.00 a.m.	4·25	5·6	3·5	2·1
	5.00 p.m.	4·50	5·5	3·3	2·2
,, 26	10.00 a.m.	3·63	6·9	4·3	2·6
	5.00 p.m.	3·24	8·1	5·0	3·1
,, 27	10.00 a.m.	2·44	8·3	5·0	3·3
,, 28	10.00 a.m.	1·76	9·0	5·0	4·0
,, 29	10.00 a.m.	1·89	9·8	5·3	4·5
Cow no. 11. Doubtful case. Parturient paralysis.					
,, 25	10.00 a.m.	2·56	10·7	5·9	4·8
	5.00 p.m.	2·73	10·4	5·4	5·0
	10.30 p.m.	2·91	10·3	5·1	5·2
,, 26	10.00 a.m.	3·47	8·1	3·4	4·7
	3·45 p.m.	3·96	5·3	2·0	3·3
	7.30 p.m.*	3·82	4·8	1·9	2·9
	10.15 p.m.	3·55	4·7	1·8	2·9
	11.30 p.m.	3·38	4·6	1·6	3·0
,, 27	5.30 a.m.	3·20	5·6	2·0	3·6
	10.00 a.m.	3·29	5·4	1·7	3·7
	5.00 p.m.	3·38	5·7	2·1	3·6
,, 28	10.00 a.m.	3·38	6·6	2·7	3·9
	7.00 p.m.	3·73	6·8	2·8	4·0
,, 29	11.30 a.m.	3·13	8·7	4·5	4·2
	7.30 p.m.	2·29	10·1	5·3	4·8
,, 30	10.00 a.m.	1·86	11·9	5·1	6·8
Cow no. 19. Doubtful case.					
Sept. 20	9.00 a.m.	2·68	10·3	5·4	4·9
,, 21	9.00 a.m.	2·86	8·5	4·4	4·1
	4.00 p.m.	2·96	5·7	4·0	3·7
	5.45 p.m.	2·48	5·5	2·3	3·2
	8.30 p.m.*	3·16	5·4	1·3	4·1
,, 22	7.15 a.m.	4·28	4·9	1·3	3·6
	5.00 p.m.	4·12	4·8	0·5	4·3
	10.00 p.m.	4·05	5·0	1·0	4·0
,, 23	9.30 a.m.	3·94	5·9	1·1	4·8
	10.00 p.m.	3·94	7·1	2·6	4·5
,, 24	9.30 a.m.	3·75	8·2	3·5	4·7
,, 25	9.30 a.m.	2·82	10·1	4·7	5·4
,, 26	9.30 a.m.	2·60	10·7	5·2	5·5

* An asterisk indicates time of calving.

Table I (*cont.*).

		CASE HISTORIES.
Normal cases.		
(1) Cow no. 13.		
Aug. 16.	11.15 a.m.	Calved with aid. Large calf. Recovery uneventful.
(2) Cow no. 5.		
Aug. 3	12.55 p.m.	Calved normally. Afterbirth retained.
„ 4		Appetite poor.
„ 5		Afterbirth removed. Appetite remained poor for several days, but subsequent recovery was uneventful.
(3) Cow no. 9.		
Oct. 15	2.00 p.m.	Calved normally. No subsequent complications.
Milk fever cases.		
(4) Cow no. 18.		
Aug. 10	1.50 p.m.	Calved normally.
	5.00 p.m.	Unwilling to rise but not staggering.
	8.50 p.m.	Fell at sampling. Unwilling to rise.
	9.55 p.m.	Condition worse. Refused to rise.
	10.45 p.m.	Condition acute. Animal comatose.
	10.55–11.03 p.m.	250 ml. of calcium gluconate injected intravenously.
	11.15 p.m.	Blood sample taken from opposite side of neck.
Aug. 11	12.15 a.m.	Improvement.
	3.00 a.m.	Refused to rise until 7·30 a.m.
	7.30 a.m.	Tried to rise.
	10.00 a.m.	Milk fever again. 325 ml. calcium gluconate given intravenously.
	2.30 p.m.	Improvement.
Aug. 12	12.15 p.m.	Able to rise if assisted.
„ 13	10.00 a.m.	Able to rise if assisted.
„ 14	10.00 a.m.	Normal.
(5) Cow no. 7.		
July 12	1.30 p.m.	Calved normally.
„ 13	10.00 a.m.	Slight staggers.
	7.30 p.m.	Condition worse.
	10.35 p.m.	Condition acute. Injected with 250 ml. calcium gluconate.
„ 14	1.00 a.m.	Improvement.
	5.30 a.m.	Walking fairly steadily.
„ 15	5.30 a.m.	Normal.
(6) Cow no. 15.		
July 19	10.00 a.m.	Fell when bled. Difficulty in rising.
	1.30 p.m.	Normal calving.
	7.45 p.m.	Fell when bled. Difficulty in rising. Staggering.
	11.30 p.m.	Unable to rise. Not known to have risen. Hourly examinations. Condition unchanged until.
„ 20	4.00 a.m.	Condition much worse.
	5.15 a.m.	Symptoms acute.
	5.40 a.m.	400 ml. calcium gluconate injected subcutaneously.
	8.45 a.m.	Marked improvement.
	4.45 p.m.	Able to rise and stand.
	9.45 p.m.	Able to walk but unsteady.
„ 21	10.00 a.m.	Gait almost normal.
	11.00 a.m.	Afterbirth removed. Subsequent recovery normal.
Doubtful cases.		
(7) Cow no. 12.		
July 22	8.45 a.m.	Calved normally.
„ 23	10.00 a.m.	Difficulty in rising but not staggering. No faeces passed since calving. Appetite poor.
„ 24		Given 1·5 pints linseed oil.
„ 25		Very little faeces passed. Given 1 pint linseed oil. Ate a little grass.
„ 26		Appetite still only fair, but improving.

Table I (*cont.*).

(8) Cow no. 11. *Post partum* paralysis.		
July 26	6.00 p.m.	Calved normally. Unable to rise afterwards.
	10.30 p.m.	No evidence of milk fever, but unable to rise. Inversion of uterus. Afterbirth removed, uterus irrigated and returned. Vulva stitched.
	11.30 p.m.	1 oz. chloral hydrate administered. Tongue flaccid. Possibility of milk fever. 250 ml. of calcium gluconate injected subcutaneously.
„ 27	3.15 a.m.	Marked improvement in carriage of head.
	5.30 a.m.	Uterus inverted between stitches in vulva, returned. Still unable to rise.
Aug. 7		Still unable to rise. Animal slaughtered.
9) Cow no. 19.		
Sept. 21	8.15 p.m.	Calved normally.
„ 22	5.00 p.m.	Fell when bled. Afterbirth removed.
	10.00 p.m.	Steadier.
„ 23	2.30 a.m.	Almost normal.
„ 24		Normal.

RESULTS.

Serum-magnesium.

Normal cases. Of the three animals which calved normally, two (Cows nos. 9 and 13) showed a rise in serum-Mg at or about the time of calving similar to that recorded by Allcroft and Godden [1934], whilst in the third case there was no marked rise.

Milk fever cases. The three animals which showed definite symptoms of milk fever all gave a rise in serum-Mg at the time of calving, of the order generally found in normal cases. In two of the cases there was then a slight fall in the serum-Mg which was followed by a very definite rise persisting throughout the period of milk fever. In the third case the fall was not noted but the rise continued until the clinical symptoms abated. It is interesting to note that this rise, which persisted after the injection of calcium gluconate, is not in keeping with the results usually found with rising serum-Ca. In general a rise in serum-Ca is accompanied by a fall in serum-Mg and this holds for cases of normal parturition and for the milk fever cases until the condition becomes acute. Following the injection of calcium gluconate in these latter cases however the consequent rise in serum-Ca is not accompanied at first by a fall in serum-Mg, the latter only occurring when the condition of the animal has almost returned to normal.

Doubtful cases. In these three cases there was found the usual rise in serum-Mg at calving. In the two cases in which the animals received no subsequent treatment there was a further rise similar to that occurring in the milk fever cases. The results for the third case (no. 11) are of doubtful value owing to the varied treatment which the animal received (*vide* case history).

Total serum-calcium.

In all cases, whether milk fever occurred or no, the changes in total serum-Ca were in general accord with those already recorded in the literature. An examination of the total serum-Ca figures for the period around parturition shows however that, in all the cases where milk fever subsequently occurred and in the doubtful cases, the fall in serum-Ca just before and at calving was very much steeper than in the case of the normal animals. It appears that this steep fall is probably a beginning of the syndrome of milk fever, but the development of the latter into an acute condition is dependent upon the simultaneous low values for both adsorbable calcium complexes and non-adsorbable calcium, as discussed below.

Calcium complexes adsorbable on barium sulphate.

Normal cases. The values were at their lowest about the time of calving, the lowest value being 3·5 mg. per 100 ml., and they quickly rose again to normal, except in the case of Cow no. 5, where there were the complications of loss of appetite and retained afterbirth.

Milk fever cases. The fall in the complexes accounts for the major part of the fall in total serum-Ca in cases of milk fever. With a pre-parturient level of about 5·5 mg. per 100 ml., minima as low as 0·5 mg. were found. These animals showed a much steeper fall in adsorbable calcium complexes just before calving than did normal animals. The values remained uniformly low during milk fever, persisting at this level until definite improvement occurred.

Doubtful cases. In these three cases the complexes showed similar diminutions to those of the milk fever cases. In the case of Cow no. 11 the onset of paralysis made it difficult to determine whether or no she was going to develop milk fever. In the case of Cow no. 12 the difficulty in rising coincided with the minimum value of 0·9 mg. per 100 ml. and in the case of Cow no. 19 the value of 0·5 mg. per 100 ml. coincided with the animal falling at blood sampling.

Non-adsorbable calcium ("protein-bound" Ca and Ca ion).

Normal cases. In two cases the non-adsorbable Ca was lower at calving than before, while in the third case there was a slight rise.

Milk fever cases. The non-adsorbable Ca in milk fever cases showed greater depression than in normal cases. In Cow no. 7 the acute symptoms did not coincide with the period of greatest depression of the non-adsorbable calcium, but only occurred when this fraction underwent a second depression, which corresponded with the minimum value, namely 0·5–0·6 mg. per 100 ml. of the adsorbable complexes. In Cow no. 15 the acute symptoms occurred when the non-adsorbable calcium reached a minimum, and the adsorbable complexes were at a low level. In Cow no. 18 there were two periods when the symptoms became acute and each of these occurred when both the adsorbable and the non-adsorbable fractions were simultaneously at low levels.

Doubtful cases. In one case, Cow no. 19, the unsteady gait and difficulty in rising, usually associated with milk fever occurred at a higher level of non-adsorbable calcium, *i.e.* 4·3 mg. per 100 ml., than might have been expected. In the case of Cow no. 12, the period of depression of this fraction was associated with loss of appetite and constipation. Cow no. 11 showed a low value, but her condition made accurate diagnosis impossible.

DISCUSSION.

The striking diminution in adsorbable calcium complexes which was found to occur in milk fever should be considered in the light of the results of Benjamin and Hess [1933]. They found that in low phosphorus type rickets the adsorbable complexes were below normal and that this was coincident with low serum-inorganic phosphorus. Later work by Benjamin [1933] showed that the ultrafiltrable calcium complex contained Ca^{++}, $PO_4^{\equiv}$ and HCO_3^- ions. Since this is so, any decrease in the amount of the ultrafiltrable calcium complex would result in a corresponding decrease in the inorganic P, unless more phosphorus were secreted into the blood stream to make good this deficit. While normally the inorganic P of the blood varies inversely with the total Ca of the serum, at parturition both values fall simultaneously. The low inorganic P values found in milk fever by Fish [1930] and other workers can be interpreted therefore as being caused by the tremendous reduction in the $Ca_x(PO_4)_y.(HCO_3)_z$ complex.

The parturient cow has considerable demands for calcium owing to the sudden onset of lactation which must be met by mobilisation of calcium from the bone reserves. Benjamin [1933] has shown that the ultrafiltrable calcium complex is removed from normal serum by calcifying cartilage and thus it is of interest to note that the failure of the parturient cow to mobilise her calcium reserves in milk fever should be associated with a decrease in the calcium complexes, of which the ultrafiltrable complex forms the greater part. Further work in progress has shown that the ultrafiltrable portion constitutes about 80 % of the total adsorbable complexes.

The connection between the parathyroids and low serum-calcium has led some workers to believe that these glands are intimately connected with milk fever. The main objection to this theory has been the low serum-inorganic P of milk fever cows. It appears that the failure to mobilise calcium, whether directly dependent on the parathyroids or no, is indirectly responsible for the failure to mobilise phosphorus.

The fluctuations in non-adsorbable calcium are in agreement with the results of Seekles *et al.* [1932] and Sjollema and Seekles [1930]. They found that the decrease in the Ca ion in milk fever to an average level of 0·44 mg. per 100 ml. ran closely parallel with the increase in severity of the symptoms. The decrease which we have found in total non-adsorbable calcium is, however, more than sufficient to account for the drop in Ca ion. From the work of Stewart and Percival [1928] the Ca ion seems to be mobilised from the "protein-bound" calcium although to what extent the organism is capable of performing this is unknown. If this process takes place, as apparently it does, during milk fever, it seems that the newly formed Ca ion disappears from the blood as fast as it is formed.

It appears that the symptoms of true milk fever only become acute when the values for both the adsorbable calcium complexes and the non-adsorbable calcium are simultaneously at low levels and near to their minima. A reference to Table I will show that in some cases the value of the non-adsorbable fraction had begun to rise slightly before the adsorbable fraction had reached its minimum and *vice versa*, and that only when the low values coincided did the condition become acute. The adsorbable calcium complexes fell to a level, in one case, of 10 % of their original value. The fall in non-adsorbable Ca was much less, the lowest value obtained being about 40 % of its original value. In the doubtful cases (Cows nos. 12 and 19) the adsorbable complexes were at their minima at the time when the non-adsorbable calcium showed maximum values and only the preliminary symptoms of milk fever appeared.

A point of interest in connection with the injection of calcium gluconate may best be illustrated by reference to the results for Cow no. 18. 12 minutes after the completion of the injection of 250 ml. of calcium gluconate at 10.55–11.03 p.m. on August 10th the total serum-Ca had risen from 4·5 to 6·6 mg. per 100 ml., the non-adsorbable calcium had risen from 2·9 to 4·7 mg., whereas the adsorbable complexes had only risen from 1·6 to 1·9 mg. Similar but less marked results were obtained on August 11th. A rough calculation based on the amount of calcium gluconate injected and the rise in total serum-calcium on August 10th shows that only about 15 % of the calcium injected could be found in the serum 12 minutes after injection.

The serum-Mg values are of particular interest and especially those maxima which occurred several hours after the crisis of the milk fever condition. As pointed out on page 452, the serum-Mg, subsequent to calcium gluconate injection, rose at the same time as the serum-Ca rose and this rise persisted until the milk fever conditions subsided. It does not appear that this parallel rise is necessarily consequent upon the injection since in two of the doubtful cases (Cows nos. 12 and 19) it also occurred without any injection of calcium gluconate. At the moment we are unable to offer any explanation of these changes in the level of serum-Mg in milk fever cases. The possible relationship between these results and those recorded by Bell and Morris [1934], indicating the persistence of an oxytocic substance in the blood of cows suffering from milk fever, has been considered, but the two sets of data run by no means parallel.

SUMMARY.

1. Values are recorded for serum-Mg, serum-Ca and calcium partition in the blood of dairy cows at parturition and in milk fever.

2. The acute symptoms of milk fever are preceded by a much steeper fall than usual in the total serum-Ca before and at calving and arise when there is a simultaneous fall in both the adsorbable calcium complexes and the non-adsorbable calcium ("protein-bound" Ca *plus* Ca ion).

3. The fall in adsorbable Ca complexes accounts for the major part of the fall in total serum-Ca in cases of milk fever.

4. During the recovery stage of milk fever serum-Mg and serum-Ca rise together.

5. A possible explanation is offered of the low values for serum-inorganic P in milk fever.

We wish to acknowledge our indebtedness to Mr W. Thomson for his care of the animals and for assistance in the blood sampling.

REFERENCES.

Allcroft and Godden (1934). *Biochem. J.* **28**, 1004.
Bell and Morris (1934). *Brit. J. Exp. Path.* **15**, 143.
Benjamin (1933). *J. Biol. Chem.* **100**, 57.
—— and Hess (1933). *J. Biol. Chem.* **100**, 27.
Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.
Denis (1922). *J. Biol. Chem.* **52**, 411.
Fish (1930). *XIth Internat. Vet. Congress*, London.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Holzapfel (1934). *Onderstepoort J., Vet. Sci., Anim. Ind.* **2**, 115.
Little and Mattick (1933). *Vet. Rec.* **13**, 1091.
—— and Wright (1925). *Brit. J. Exp. Path.* **6**, 129.
Seekles, Sjollema and van der Kaay (1932). *Biochem. Z.* **249**, 424.
Sjollema and Seekles (1930). *Biochem. Z.* **229**, 358.
Stewart (1933). Private communication.
—— and Percival (1928). *Biochem. J.* **22**, 559.

LII. A STUDY OF THE UNSAPONIFIABLE FRACTION OF WHEAT GERM OIL WITH SPECIAL REFERENCE TO VITAMIN E.

By JACK CECIL DRUMMOND, ELEANOR SINGER
and ROY JAMES MACWALTER.

From the Department of Biochemistry, University College, London.

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Previous chemical studies of vitamin E have shown it to be a substance which can readily be concentrated in the unsaponifiable fraction of certain oils. The properties of these concentrated preparations indicate that the active substance possesses a relatively high degree of stability. The original investigations of Evans and Burr [1927, 1] showed that the vitamin is not readily destroyed by aeration at 100°, treatment with 20 % hydrochloric acid, subjection to temperatures up to 250° or hydrogenation with palladium at 75°. Exposure to strong ultra-violet radiation seemed to cause slight inactivation after 20 minutes, whereas after 45 minutes much activity was lost. Treatment with alcoholic hydrochloric acid or bromination in glacial acetic acid caused inactivation and, more surprisingly, evidence that acetylation was also destructive was obtained.

Little had been added to this knowledge of the chemical behaviour of vitamin E concentrates until Olcott and Mattill recently began to report the results of their investigations [1934; Olcott, 1934, 1, 2].

In the main the observations of Evans and Burr were confirmed. A striking exception was a failure to bring about inactivation by acetylation and an important addition was successful reactivation of chlorinated and brominated material by boiling with zinc dust and hydrochloric acid in methyl alcoholic solution. Destruction by ozone, perbenzoic acid, potassamide and, somewhat unexpectedly, potassium ethoxide was recorded.

Investigators are agreed that the vitamin is volatile. Evans and Burr found on refractionating that activity was associated with material collected in the narrow range 225–30°/0·01 mm., but Olcott and Mattill recorded a much greater volatility (190–220°/0·1 mm.).

An important question concerns the absorption bands shown by concentrates of vitamin E. Evans and Burr remarked "these measurements did not point to any definite curves for vitamin A or E which would help in following the concentration of the substance". Broad but diffuse bands, becoming much sharper at the temperature of liquid air, were however detected by Bowden and Moore [1933, 1] and additional maxima were recorded by Morton and Edisbury [1933]. Correlation of these data with biological activity was first attempted by Bowden and Moore [1933, 2] who pointed out that some part of the absorption in the range 320–285 $m\mu$ might be related to vitamin E.

Edisbury [1933] also reported a detailed consideration of the spectroscopic data obtained from the examination of vitamin E concentrates, with the object of tracing a correlation with the chemical behaviour of the vitamin.[1] He also reached the conclusion that the bands in the region indicated by Bowden and Moore should be further studied and drew attention to the three maxima, 291, 271, and 256·5 $m\mu$. The work of Olcott and Mattill [1934] tended however to weaken the belief that the vitamin might be a substance showing marked selective absorption in the ultra-violet, because their highly concentrated fractions showed only general absorption. Recently, however, a preliminary communication by Martin *et al.* [1934] announced that a well-defined absorption band was exhibited by a vitamin E concentrate. The band in question had a maximum at 294 $m\mu$; the strongest absorption recorded being $E^{1\%}_{1\,cm} = 27$. This material gave a positive biological test on one female rat when a single dose of 16 mg. was administered.

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[1] We are greatly indebted to Dr R. A. Morton for his kindness in bringing this report of Dr Edisbury's work to our attention and for putting unreservedly at our disposal the results of his own spectrographic examinations of vitamin E concentrates.

Olcott [1934, 2] has also recorded this band in the examination of concentrates from wheat germ oil and cottonseed oil. Whereas Martin and his colleagues are undecided whether the band can be ascribed to the vitamin or to accompanying substances, Olcott concludes tentatively that it is not related directly to the vitamin because certain treatments ($AgNO_3$, acetylation) change or obliterate the band but do not destroy biological activity.

Chemical analyses of biologically active fractions have been made but the results have been put forward with reservations regarding the homogeneity of the material. These figures will be referred to later.

EXPERIMENTAL.

The raw material employed for the investigation now being recorded was the unsaponifiable fraction of wheat germ oil. The oil was prepared by cold extraction of a commercial wheat germ preparation (Bemax) with purified trichloroethylene. Ordinary commercial wheat germ is seldom a good raw material because it usually contains a large proportion of bran and other milling products and, moreover, may often be badly rancid. These facts are often ignored. On the one hand, some recorded estimations of the vitamin B content of wheat germ are low because of the unsuspected presence of bran; on the other, it is reasonable to attribute the low vitamin E activity of some preparations of wheat germ oil to the post-milling changes which rapidly occur in the crushed embryo. In this connection the work of Evans and Burr [1927, 2] on the influence of rancidity on vitamin E is of interest. The preparation (Bemax) chosen for this experiment is practically pure germ, has a negligible degree of free acidity and no trace of other products associated with rancidity.

The germ yielded 7·2 % of oil possessing the following characteristics:

Sap. value	182
Iodine value	131
Refract. index (20°)	1·4773
Unsap.	5·76 %
Iodine value of unsap.	97·6

The oil was found to be biologically active in daily doses of 23 mg. In our experience extraction with ether may sometimes cause inactivation. One sample prepared from the same bulk of germ failed to protect 5 rats from resorptions when given in daily doses of 46 mg. Preparation of the unsaponifiable fraction was made by treatment with boiling alcoholic KOH. Two saponifications of 30 minutes' duration were found to serve best. In the light of our experience it is surprising to learn that Olcott [1934, 2] has found inactivation after treatment with potassium ethoxide.

The unsaponifiable fraction thus obtained was, at room temperature, a hard yellow wax. It was found to be active in doses of 2·5 mg. It usually contained about 60 % of sterols precipitable by digitonin.

The bulk of the sterols was removed by cooling a saturated solution in methyl alcohol to $-25°$ by the direct addition of powdered CO_2. This treatment effectively removed between 80 and 90 % of the sterols present. The mother-liquor from the crystallisation of the sterols yielded on removal of the methyl alcohol a red-orange coloured, soft wax.

Crude sterol fraction.

As was suspected from reference to the observations of Anderson and Nabenhauer [1924, 1], the greater part of the crude sterol mixture was found to consist of sitosterol and dihydrositosterol. Using their method of separation [1924, 2; Anderson *et al.* 1926–27], a preparation of the saturated constituent was obtained possessing the following characteristics: M.P. 143–5°; $[\alpha]_D^{18}+21{\cdot}9°$; acetate, M.P. 141°; $[\alpha]_D^{18}+13{\cdot}29°$. All the sterol fractions showed absorption bands at 261 $m\mu$ and usually well-marked bands at 254 and 244 $m\mu$ in addition to the triple system characteristic of ergosterol. Similar findings have been reported by Morton and Edisbury [1933] and by Edisbury [1933]. It is probable that certain of these bands are related to ergosterol D, but unidentified sterols may also be concerned.

Fractional adsorption experiments.

Preliminary experiments having shown that more promising fractionation of the unsaponifiable material after removal of the greater part of the sterols was effected by adsorption on aluminium oxide than by distillation in high vacua or by partition between solvents, the former method was adopted. A typical experiment is described. 33 g. of the viscous red-orange oil obtained after removal of the crude sterols were dissolved in 500 ml. light petroleum. A small amount of dark brownish red material remained undissolved and was filtered off. More of this dark red material separated from the petroleum solution on standing overnight. The combined weight of the two insoluble fractions was 0·67 g. It proved to be slightly impure lutein (xanthophyll). Recrystallisation from methyl alcohol gave typical crystals, M.P. 194·5°.

The material soluble in light petroleum was passed slowly through a column of "Aluminium oxydatum anhydricum, nach Brockmann" (Merck), long-continued washing with the solvent being maintained. Two fractions of unadsorbed material were separated. The first represented that which passed through the column without any delay (fraction *A*). The second corresponded with a pale yellow coloured zone which slowly passed down the column as the washing proceeded. Eventually this pigmented substance passed out into the receiving flask completely, the washings once again becoming colourless (fraction *B*).

At this stage the experiment was usually discontinued and the column of adsorbent was broken up into four sections. The uppermost layer containing the most strongly adsorbed substance was of a deep brownish yellow tint (fraction *F*). Next came a paler, sandy coloured zone (fraction *E*), below which was another coloured region but definitely darker in tint (fraction *D*). At the bottom of the column came a deep zone showing in general little colour but marked by more than one indefinite band of slightly more pronounced yellow or sandy colour (fraction *C*).

It must be emphasised that this description refers only to one experiment. In every case the behaviour was similar so far as the separation of fractions *A* and *B* was concerned but the appearance of the column itself at the end of the experiment was not always the same.

To what causes these differences, not always of a minor character, are to be ascribed we do not know, but with experience it was found possible to recognise and separate the important sections of the column. The fractions of oxide were separately eluted with 80/20 methyl alcohol-ether.

The amount and characteristics of the fractions obtained in the actual experiment we have described are given below in Table I.

Table I.

Fraction	Appearance	Wt. (g.)	Iodine value	Biological activity	Spectroscopic examination max. at $m\mu$
A	Clear, pale lemon-yellow oil	6·23	64	—	388, 362, 337, 323, 289
B	Orange oil	3·97	235	—	485, 453, 362, 320, 289
C	Rich orange oil	6·97	200	Active 1 mg., slightly active 0·2 mg.	492, 303, 295
D	Soft red wax	6·16	216	Active 2 mg., slightly active 1 mg.	475, 445, 295
E	Dark brown-red crystalline mass	4·60	101	Slight activity 2·5 mg.	344, 327, 303, 297, 284, 273
F	Dark red, thick oil containing crystals	3·34	105	—	475, 445, 416, 347, 335, 320, 297, 284, 273

Fraction A. Superficial examination of this mixture suggested that it consisted for the greater part of hydrocarbons. It contained material precipitated by digitonin, the amount usually being about 3·5 %. This was removed before further fractionation was attempted by passing again through aluminium oxide. The latter treatment removed by adsorption a small amount of material with a high iodine value (see fraction *B*) and gave clear, pale yellow oils with iodine values in the range 40–50. This material seeming to be suitable for purification by distillation the latter was carried out (*a*) by the usual procedure at a pressure of 0·05 mm. and (*b*) by use of the Burch [1929] molecular still.[1] Even at the higher pressure (*a*) the material distilled readily. A typical distillation gave the following fractions:

(*a*) 2·1 g. distilled at 0·05 mm.

Temp. range	Wt. of fraction g.	Iodine value	Refractive index 18°	C %	H %
122–148°	0·861	39·4	1·5002	86·49	12·35
148–165°	0·779	37·7	1·5062	86·78	12·42
165–200°	0·359	46·7	1·5088	86·09	12·05
above 200°	0·079	69·0	—	—	—

(*b*) A fractionation of 4·35 g. in the molecular still (pressure *ca.* 0·00001 mm.).

Temp. range	Wt. of fraction g.	Iodine value	Refractive index 18°	C %	H %
55–69°	1·75	43·7	1·5009	86·64	12·16
69–120°	1·25	73·0	1·5059	86·06	12·44
above 120°	0·70	71·0	—	—	—

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[1] We are much indebted to Dr F. H. Carr and Mr Jewell of B.D.H. Ltd. for carrying out this and a number of other fractionations in the molecular still. We greatly appreciate their valuable and ready help.

Inspection of these figures suggests a relatively low-boiling hydrocarbon contaminated with a less volatile oxygen-containing impurity possessing a higher degree of unsaturation and a higher refractive index. We have succeeded in obtaining after more than one distillation preparations of the former which gave satisfactory analysis.

The best material had the following properties:

B.P.	120–140° (0·05 mm.)	
Iodine value	42	
C %	87·02, 87·05	
H %	12·82, 12·96	
Mol. wt. (camphor)	258, 249	
Refract. index 18°	1·5009	
Absorption bands	336 $m\mu$	medium intensity
	326·5 ,,	very narrow
	301 ,,	inflection
	288 282 277 ,,	very weak and narrow
	257 ,,	broad and strong

The analyses would agree with a formula such as $C_{18}H_{32}$ (C, 87·09; H, 12·91 %).

Some or all of the members of this group of absorption bands appear to be always shown by these hydrocarbon fractions and it is of interest to note that they were also exhibited by a similar fraction obtained from olive oil by Thorbjarnarson and Drummond [1934]. It is possible that those with maxima at 336, 326·5 and 301 $m\mu$ may be associated with dihydroergosterol, although these bands do not correspond closely with the published figures.

Enough of this liquid hydrocarbon has not yet been available to enable us to examine its properties more fully.

In view of the discovery in fraction *B* of a highly unsaturated hydrocarbon of the squalene type it was thought at one time that the iodine value of preparations from fraction *A* might be due to contamination with this substance. An effort was made to separate the suspected contaminant by bromination and by treatment with HCl (see fraction *B*), but without appreciable success. Only very minute amounts of solid derivatives separated and on removing the halogens from the residues the iodine values remained in the neighbourhood of 35–40.

It would be inadvisable to comment further on this compound until larger quantities have been prepared and examined. The fraction was inactive as a source of vitamin E.

Fraction B. The constituents of this fraction passed slowly through the adsorbing column, visibly as a pale yellow zone, during the washing with light petroleum. The pigment was characterised by absorption bands in the visible spectrum at 453 and 485 $m\mu$. The position of these bands and the behaviour during adsorption suggest that the colour is due to kryptoxanthin[1] [Kuhn and Grundmann, 1933]. Absorption in the ultra-violet zone was indicated by maxima at 362, 325 and 289 $m\mu$. The intensities were usually of approximately the same order as that obtained in the examination of fraction *A*. The significance of these bands is as yet unknown.

This fraction was characterised by high iodine values, the range encountered in different experiments being 215–266. This fact, together with the low adsorbability of the fraction, suggested the presence of a highly unsaturated hydrocarbon. Attempts were made therefore to prepare halogenated derivatives. Bromination in dry ether at 0° gave a moderately good yield of a white micro-crystalline bromide resembling the bromides of the squalene type of hydrocarbon. This derivative darkened at 165° and showed definite signs of decomposing at 180°. It contained 69·2 % of bromine. Treatment with dry HCl gas in acetone at $-10°$ gave crystalline derivatives, the form of which usually recalled the prismatic plates of squalene hydrochloride. In a few cases however the typical plates did not appear and the form was that of bunches of very small fine needle-shaped crystals, more like the hydrochloride of the hydrocarbon isolated by Channon and Marrian [1926] from pig liver fat. Sometimes both types of crystals have appeared. Preparations of the hydrochloride recrystallised from acetone showed melting-points within the range 107–116°, but insufficient was available to enable an adequate fractionation to be carried out. Recent evidence [Thorbjarnarson and Drummond, 1934] supports the belief of earlier investigators that several isomeric squalene hydrochlorides with different melting-points exist.

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[1] In a preliminary report of this work the pigment was described as β-carotene, the absorption bands of which correspond closely with those of kryptoxanthin [Drummond *et al.*, 1934].

The analytical figures for the hydrochlorides are also unsatisfactory. Squalene hydrochloride, $C_{30}H_{50}.6HCl$, has 33·9 % Cl; the hydrochloride of the liver hydrocarbon is probably $C_{45}H_{78}$, 8 HCl containing 31·2 % Cl [Channon *et al.*, 1934]. Various preparations of our hydrochloride gave 31·34, 31·39, 31·99, 32·08, 32·24, 32·79, 33·34 % Cl.

Until material is available for a more thorough study it is unwise to draw conclusions from these figures but they suggest the possibility that more than one hydrocarbon of the squalene type is present. It is not unreasonable to think that the liver hydrocarbon might be present in plant materials, particularly since squalene has recently been identified as an important constituent of olive oil.

Analysis of the crude fraction *B* having shown that considerable amounts of oxygen-containing substances were also present, even after removal of all material precipitable by digitonin, efforts were made to separate them from the hydrocarbon by the use of solvents and by fractional distillation. Neither was successful. Fractions were obtained with higher iodine values, the highest being 306 (squalene has I.V. 371), but analyses and the yields of hydrochloride and bromide showed that no effective concentration had been achieved.

Since it appeared that at least one of the oxygen-containing substances was highly unsaturated attempts were made to separate it on the assumption that it was alcoholic in character by acetylation and benzoylation. Evidence that reaction occurred was obtained, but no real success in separation was attained; once again our efforts being limited by the small scale on which we were obliged to work. Fraction *B* was found to be biologically inactive as a source of vitamin E.

Fraction C. The material yielded by elution of the lower zone of the adsorption column was in all experiments a deep orange-yellow viscous oil. The amount was usually of the order of one-fifth of the original material submitted to adsorption. When sterols had not been completely removed before the adsorption was performed as much as 15 % of material precipitated by digitonin was found in this zone. In such cases it was quantitatively removed before further fractionation was attempted. The sterol-free material behaved on general examination as if it were composed to a large extent of unsaturated alcohols of high molecular weight. The degree of unsaturation was indicated by iodine values ranging from 170 to 200. This fraction invariably showed a well-marked absorption band with a maximum at 294 $m\mu$ (Curve *A*, Fig. 2), to which we are inclined to attach importance. Related to this maximum is a minimum displayed at 267·5 $m\mu$, but in the examination of some preparations, particularly in the early stages of fractionation, this may be masked by general absorption. For a typical preparation of sterol-free material the measurements gave $E^{1\%}_{1\,\mathrm{cm}}$ 294 $m\mu = 27{\cdot}8$;

$267{\cdot}5\ m\mu = 17{\cdot}3$. Definite biological activity is found in this fraction, a daily dose of 0·2 mg. producing satisfactory litters. Since 1 mg. of the material before adsorption exerted about the same biological activity this indicates satisfactory concentration without significant loss.

Adsorption having proved so helpful and other methods of fractionation having given such disappointing results it was decided to carry the concentration as far as possible by use of aluminium oxide. Fraction *C* was therefore readsorbed from light petroleum solution, on appropriately smaller columns of the oxide. One unexpected observation was made. Whereas in the initial adsorption the biological activity was associated with the fractions more lightly adsorbed, in subsequent readsorptions it was usually found in fractions located higher up the column. After a third readsorption it appeared to be almost wholly concentrated in the top zone of the oxide and held there in spite of prolonged washing with solvent (see Fig. 1). Without doubt the position taken up by any substance in these adsorption columns is much influenced by the presence of other substances. In our experience it was the removal of the highly unsaturated substance described as being found in fraction *B* which rendered the vitamin more readily adsorbed by the oxide.

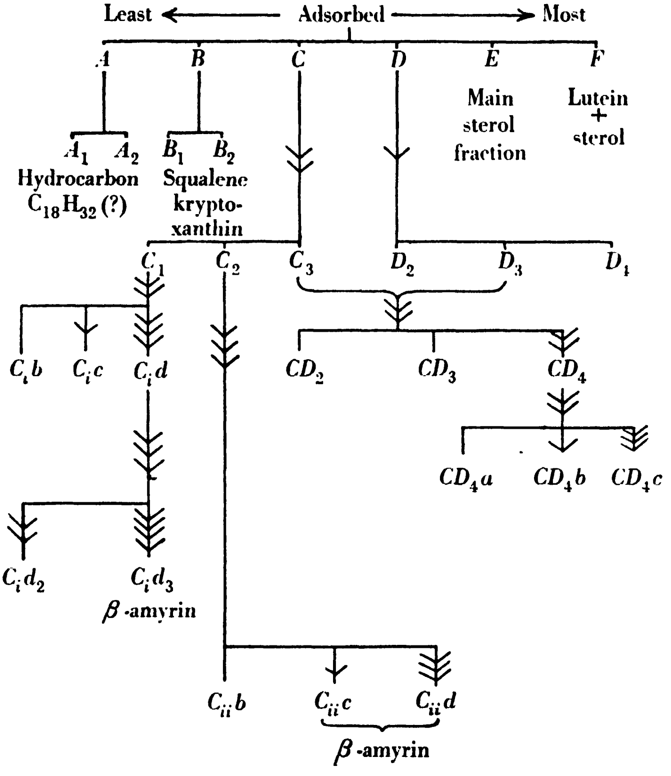

Fig. 1. General scheme of fractionation of wheat germ oil unsaponifiable. The "arrow-heads" indicate the fractions which contained the substance responsible for the 294 $m\mu$ band. An attempt to indicate the quantitative distribution has been made by using one to four "arrow-heads". In general the distribution of the vitamin is similar.

Biological activity having also been detected in fraction *D* (see later) and refractionation of this material being decided upon, it became necessary to combine certain fractions from the main fractions *C* and *D*. The scheme of separation is shown in Fig. 1.

From Fig. 1 it will be seen how curiously the active material changes in its behaviour from being adsorbed at the bottom of the column to being readily held up by the uppermost layers of the oxide.

At the stage of fractionation represented by the fractions 75 $C_i d$ and 75 $C_{ii} d$, the biological activity was found to be represented by the minimum daily dose of 0·1 mg. This corresponds satisfactorily with the factor of concentration represented by the material yields. Further attempts at concentration were disappointing. Fractions 75 $C_i d_2$ and 75 $C_i d_3$ were not found active in 0·1 mg. but a hydrogenated preparation of the former was found active in doses of 0·2 mg.; unfortunately, this material was not tested in smaller doses.

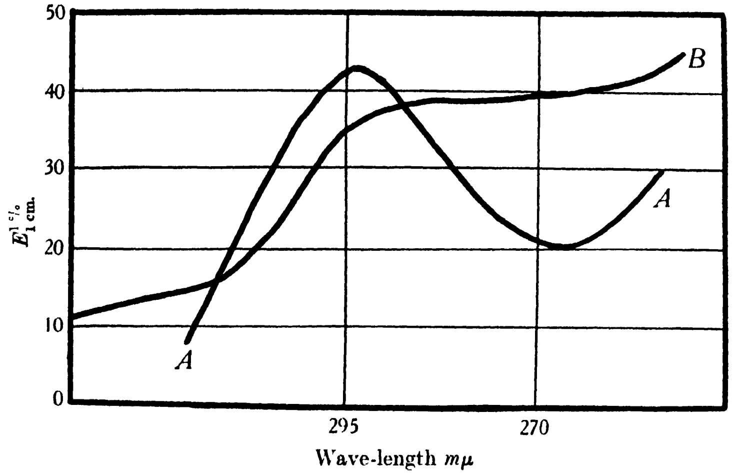

Fig. 2. Influence of 12 hours' short range irradiation by a mercury vapour lamp on the absorption band of an active vitamin E concentrate. Little change in the absorption at the maximum at 294 *mμ* but a great decrease of the "persistence" are regarded as evidence of considerable reduction in the concentration of the substance responsible for the band (see p. 465). (*A*) Original material. (*B*) Irradiated material.

A general parallel was noted between biological activity and the intensity of absorption in the ultra-violet region at 294 *mμ*, particularly when this was measured in relation to the minimum at 267·5 *mμ*.

In attempting to find a correlation between the biological activities of our concentrates and their absorption measurements we found it important to consider this relation rather than the individual values for $E^{1\%}_{1\,\mathrm{cm.}}$. In general, the substance responsible for the selective absorption at 294 *mμ* will be contaminated by other absorbing materials. Removal of these latter often causes a decrease in the measured maximum of absorption, but also causes a decrease (to a proportionately greater extent) in the absorption at the minimum of the band.

We have therefore been concerned with two criteria in considering the effect of concentration of the substance responsible for the band: (i) increase in absorption at the maximum and (ii) "persistence", which we describe as the ratio of maximum to minimum absorption. We are inclined to consider that this ratio provides a better estimate of the effect than the difference between maximum and minimum absorption.

Certain data for four of the more powerfully absorbing fractions obtained are set out in Table II.

Table II.

Fraction	Iodine value	Absorption $E^{1\%}_{1\,\text{cm.}}$ 294 $m\mu$	Absorption $E^{1\%}_{1\,\text{cm.}}$ 267·5 $m\mu$	Ratio 294/267·5	Optical rotation approx. 1·5 % in benzene $[\alpha]^{18°}_{D}$
75 $C_{ii}d$	167	42·6	20·0	2·13	+20·0°
75 $C_{i}d_{3}$	177	39·6	20·4	1·96	+16·2
75 $C_{i}d_{3b}$	164	40·9	12·8	3·18	+22·6
75 $C_{i}d_{3c}$	141	45·0	17·6	2·56	+18·0

Investigation of the nature of these active fractions led in due course to acetylation which immediately revealed that they were not homogeneous. Variable amounts of a beautifully crystalline acetate were obtained, some of the higher yields being as much as 15 %. The acetate was slightly soluble in methyl and ethyl alcohol, more readily in ether and chloroform. Recrystallised from acetone or ethyl alcohol it melted sharply at 236°. (Found: C, 81·59, 81·24; H, 11·10, 11·26 %; mol. wt. 442, 455; $[\alpha]^{18°}_{D}+97·3°$.) Determination of acetyl value showed the presence of one acetyl group. The parent alcohol crystallised in fine needles from ethyl alcohol, M.P. 190°. (Found: C, 84·57, 84·52; H, 11·66, 11·70 %; mol. wt. 427, 430; $[\alpha]^{18°}_{D}+97·7°$.) It appeared certain from this data that the substance was β-amyrin, $C_{30}H_{50}O$.[1] Mixed melting-point determination with pure specimens of the parent substance and the acetate showed no depression. Spectroscopic examination showed that β-amyrin exhibits only very slight absorption in the vicinity of 284 $m\mu$ ($E^{1\%}_{1\,\text{cm.}}=1·0$) so that this substance is not responsible for the selective absorption shown by the active fraction. The acetate is no more transparent. It was also without biological activity in doses of 0·1 mg. daily. It is as yet too early to dismiss the possibility that β-amyrin is related to the vitamin E activity but animal tests on this substance itself have given negative results.

Acetylation of our active fraction also threw light on another interesting matter concerning the vitamin. It will be recalled that other investigators are not in agreement regarding the effect of this treatment. Olcott did not confirm Evans and Burr's observation that destruction of biological activity followed treatment with acetic anhydride. He noted, however, that it caused a change of the absorption in that the maximum moved from 294 to 281 $m\mu$. He does not mention the relative intensities but he is inclined to use these observations as support for concluding that the former band is not connected with the vitamin content.

We have found that selective absorption disappears on acetylation and reappears on hydrolysis but biological activity is unaffected. We have as yet insufficient data to give us confidence, but we are inclined to believe that immediately after hydrolysis the 294 $m\mu$ band may reappear with an intensity greater than that shown by the material before acetylation. The position of the band and its intensity are not incompatible with the substance being a polycyclic ketone. Apart from this question the disappearance and regeneration of the band suggest that the responsible structure may be one capable of undergoing tautomeric change.

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[1] Mr F. A. Askew has very kindly examined this compound by the surface film technique and is satisfied that it behaves in a manner similar to what would be expected from a very slightly impure β-amyrin [Askew, 1935].

Susceptibility to ultra-violet radiation. If the absorption band at 294 $m\mu$ is a property of the vitamin, it would be expected that radiation at the appropriate frequency would result in photochemical change, and that changes occurring in the absorption spectrum would be accompanied by corresponding changes in vitamin activity. Evans and Burr [1927, 1] found signs of loss of biological activity after short range exposure to ultra-violet radiation for 20 minutes at 0° and definite destruction after 45 minutes at ordinary temperature. Our preliminary experiments with a relatively crude preparation (55 F) indicated an even greater degree of stability. This preparation before radiation was biologically active in doses of 0·2 mg. daily. 0·33 g. of the preparation contained in a quartz flask and sealed under nitrogen was exposed to the direct rays of a mercury arc lamp (atmospheric type) at a distance of 3 inches for 13·75 hours. The activity was not completely destroyed, since 5 out of 6 rats produced litters on a daily dose of 1 mg. A dose of 0·4 mg. was inactive.

A definite change in absorption spectrum was observable. The intensity of the original material at 294 $m\mu$ was $E^{1\%}_{1\,\text{cm}} = 48{\cdot}2$ and the persistence (see p. 463) 1·74. After irradiation the intensity maximum remained constant ($E^{1\%}_{1\,\text{cm}} = 47{\cdot}8$) but the persistence had decreased to 1·33.

A more highly refined preparation (75 $C_{ii}d$), originally active in 0·1 mg. dose, lost at least half its activity after direct exposure under similar conditions for 12 hours. In this experiment the change in absorption spectrum was considerable (see Fig. 2), the band having disappeared almost completely after irradiation.

Influence of hydrogenation on vitamin E activity. Evans and Burr [1927, 1] found that wheat germ oil and extracts prepared therefrom retained their vitamin E activity after hydrogenation with palladium at 75°. Olcott [1934, 1, 2] found that active fractions withstood drastic catalytic hydrogenation at 200° and 200 atmospheres but were still unsaturated, as indicated by an iodine value of 70.

Our own experience confirms the survival of biological activity after catalytic hydrogenation but we found no difficulty in obtaining a more satisfactory uptake of hydrogen. One of our most active fractions (75 $C_{ii}d$) was reduced with palladium at atmospheric temperature and pressure, the concentrate being dissolved in absolute alcohol. In one experiment 138 mg. of this golden yellow viscous oil took up hydrogen readily under these conditions, the iodine value falling from 166 to 26 in about 5 hours with one renewal of the catalyst. Difficulty was encountered in carrying the hydrogenation further and it is possible that the residual iodine value represented an unsaturated impurity resistant to reduction. The amounts of material available did not enable us further to investigate this matter. The hydrogenated material was a soft white wax with an indefinite melting-point about 35°. The biological activity was unaffected by the reduction, good litters being obtained with a dose of 0·2 mg. The examination of the material before and after reduction showed that the absorption band had been but slightly changed, apart from a very slight shift towards the more refrangible region, the maximum and minimum being now at 296·5 and 271 $m\mu$ respectively (Table III).

Table III.

Condition	Iodine value	Absorption $E^{1\%}_{1\,\text{cm.}}$		Ratio	Positive rat dose
Original	166	294	42·6	2·13	0·2 mg.
		267	20·0		
Partly reduced	70	296	40·2	2·58	—
		268·5	15·6		
Maximum reduction achieved	26	296·5	37·8	2·52	0·2 mg.
		271	11·0		

Unfortunately, we have not had sufficient reduced material to make it possible seriously to attempt chemical fractionation but a few preliminary trials have given encouraging results.

Chemical composition of the active fractions. We have thought it worth while to make a few analyses of our most active fraction although we are fully aware that they may have been far from homogeneous. These materials are yellow and somewhat viscous oils. The analytical data are given below:

	C %	H %	Mol. wt. (camphor)	Iodine value
	80·77	11·44	460	164
	81·12	11·66	430	179
	81·16	11·56	420	166
	80·62	11·61	445	162
	80·82	11·64	—	167
Mean	80·89	11·58	439	167

The material acetylates with the introduction of one acetyl group. It will be remembered that we found that the band at 294 $m\mu$ disappeared as a result of this change and was again exhibited by the material after hydrolysis. Analyses of the acetylated product gave:

	C %	H %	Mol. wt. (camphor)	Iodine value
	78·33	10·70	480	160
	78·55	10·85	440	156
	78·36	10·41	476	—
Mean	78·41	10·65	465	158

Determination of acetyl groups.[1]

(*a*) 0·1197 g. contains 0·223 milliequiv. acid.
(*b*) 0·113 g. „ 0·230 „

On the assumption that our material is homogeneous these analytical figures would agree reasonably well with a polycyclic structure related to the sterol or amyrin class.

	C %	H %	Mol. wt.	Iodine value (3 double bonds)
$C_{28}H_{48}O_2$	80·76	11·53	416	183
$C_{30}H_{50}O_2$	81·44	11·31	442	172

It will be recalled that the analyses of the distilled fraction of Evans and Burr [1927, 1] fitted the formula $C_{36}H_{64}O_2$. Regarding the degree of unsaturation it may be noted that the volume of hydrogen absorbed was of the order required to saturate three double bonds. Again assuming homogeneity, the formulae indicate the presence of two oxygen atoms in the molecule. On the spectroscopic evidence one of these might represent a ketonic grouping capable of tautomerising and yielding an acetylated derivative on treatment with acetic anhydride. If so, the second oxygen is present either as a relatively stable ketonic group or in another form not reacting to acetylation.

Again with reservations regarding purity the view that a polycyclic ketone may be concerned is not at variance with the evidence derived from a study of surface films which is presented by Askew [1935]. His data suggest that if the substance contains two polar groups they are probably close together in the molecule.

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[1] We are greatly indebted to Dr A. H. Palmer for making these determinations.

In order to test for the presence of ketonic substances in the active concentrates two methods of fractionation were investigated. One has been devised by Girard [1933] but as the nature of his reagent has not yet been fully revealed this could not be investigated without his assistance. Dr Girard was kind enough to carry out for us an extraction depending upon the use of his "Reagent T". He informs us that under the conditions of his reaction all ketones except the "aromatic nuclear ketones" are converted into water-soluble derivatives which can be readily separated from impurities soluble in ether and other fat solvents. When ketones are thus abstracted in water-soluble form the solutions give a precipitate with mercuric iodide; the reaction being so sensitive that it will detect 1 or 2 γ per ml. of oestrone. Approximately 1 g. of sterol-free wheat germ concentrate was sent to Dr Girard for treatment. He reported that the extraction and separation of the ketonic fraction did not proceed altogether smoothly. From the behaviour with HgI_2 he was inclined to think that only small amounts of ketonic substances were present. "If the molecular weight of the ketones present is about the same as that of oestrone I would consider the total amount of the ketones as not greater than 10 to 15 mg." Actually, the extracted material, a pale yellow, soft wax, weighed 10 mg. (1·0 %).

This material gave disappointing results on spectroscopic examination for no evidence of significant concentration of the substance responsible for the 294 $m\mu$ band was obtained.

Material before treatment	Inflection 294 $m\mu$ $E^{1\%}_{1\,\text{cm.}}=46$
"Ketonic" fraction	,, 294 $m\mu$ $E^{1\%}_{1\,\text{cm}}=45$
Residue after removal of "ketonic" fraction	,, 294 $m\mu$ $E^{1\%}_{1\,\text{cm}}=30{\cdot}8$

The extracted material has not yet been tested biologically.

Another attempt was made to separate ketones by the method fully described by Beall and Marrian [1934]. This was applied to two active fractions:

(*a*) a sample of the sterol-free unsaponifiable fraction similar to that which had been treated by Dr A. Girard;

(*b*) a more highly concentrated preparation (75 $C_{,,}d$).

In both cases the yield of ketonic substances was small, whilst the spectroscopic examination gave no evidence of effective concentration (Table IV):

Table IV.

	Material	Wt. g.	$E^{1\%}_{1\,\text{cm}}$ 294 $m\mu$	$E^{1\%}_{1\,\text{cm}}$ 268 $m\mu$	Remarks
a	Before treatment	2·268	46·9	32·7	Persistence (ratio) 1·4
	"Ketonic" fraction	0·206	21·6	—	Inflection slightly better defined than in "non-ketonic" fraction
	"Non-ketonic" residue	1·997	26·8	--	Very weak inflection
b	Before treatment	0·098	42·6	20	Persistence (ratio) 2·13
	"Ketonic" fraction	0·0035	43	—	Inflection only
	"Non-ketonic" residue	0·086	35	—	Bands of low persistence

Whether these "ketonic" extracts contain a high concentration of the vitamin has not yet been ascertained, but it is difficult to reconcile the relevant spectroscopic data with the view that the active substance is a polycyclic ketone normally showing a broad strong absorption band at 294 $m\mu$.

The pigment responsible for the yellow colour of fraction *C* was not satisfactorily identified. The band at 492 $m\mu$ (alcohol) indicates a lipochrome; probably a trace of γ-carotene or rubixanthin.

The substance responsible for the maximum at 303 $m\mu$ was not traced.

Fraction D. Spectroscopic examination of this soft red wax showed the presence of a lipochrome pigment resembling lutein. The only other important absorption was at 295 $m\mu$ in the ultra-violet. Since this fraction showed biological activity its investigation proceeded in the main side by side with that of fraction *C* (see Fig. 1). It contained about 33 % of the familiar sterol mixture in all cases in which this material had not been removed before adsorption. In the examination of this fraction some evidence of the presence of phytol was obtained by the preparation of phthalates but the amounts of silver derivatives isolated were too small to purify and analyse.

Fraction E. In all cases in which sterols were not completely removed by digitonin before adsorption this fraction contained the highest proportion of these substances. The appearance of the material in such cases clearly indicated its composition, as it usually resembled a crude preparation of phytosterol coloured with a brownish yellow pigment. The latter was identified as lutein. Quantitative estimations showed from 80 to 89 % of sterol precipitable by digitonin.

By recrystallisation from methyl alcohol or light petroleum clean preparations of crystalline sterol mixtures (phytosterol) were readily made. Spectroscopic examination of this material revealed bands at 342, 328, 294, 283, 272 and 262 $m\mu$; that at 283 being strong. Edisbury [1933] has recorded rather feeble maxima at 312, 323 and 343 $m\mu$ in sterol fractions and thought they might be attributed to dehydroergosterol. Only one of these is recorded in our measurements. Other maxima are probably attributable to ergosterol. Fractional crystallisation of the sterol from methyl alcohol gave crystalline preparations from the more soluble fractions which showed clearly the ergosterol group of bands and the other triplet believed to be due to ergosterol D (254, 243 and 235 $m\mu$). In the less soluble fractions, as in the original material, general absorption tended to mask selective absorption in the shorter wave-length range.

Fraction F. This material, the most firmly adsorbed, was but superficially examined as it did not possess vitamin E activity. The pigment was almost certainly lutein. Considerable amounts of sterol (35 %) and the associated absorbing substances were also present.

Behaviour of the active fractions on distillation.

Some of the later stages of fractional adsorption having given disappointing results efforts were made to carry the concentration a step further by distillation in high vacuum. Unfortunately, the quantities of material available were so small that satisfactory fractionation could not be expected. The following results refer to the distillation of 1·35 g. of fraction 75 $C_i d_3$ which was believed to be biologically active in doses of 0·1 mg. daily. Dr Carr and Mr Jewell, who very kindly carried out the fractionation of this material in a Burch molecular still, reported that "no sign of a constant-boiling fraction could be observed".

The fractions were examined with the following results (Table V).

On the assumption that the persistence of the 294 $m\mu$ is a measure of vitamin activity fraction 2 from the distillation should have been the most potent material we have possessed. Unfortunately, the biological tests were inconclusive owing to a large proportion of the animals showing infertile matings. Two

Table V.

				$E^{1\%}_{1\ \text{cm.}}$		Ratio
Fraction	Temp.	Wt. g.	Iodine value	294	267	294/267
1	76–103°	0·3	169	13·5	8·16	1·65
2	96–127°	0·45	164	40·9	12·8	3·18
3	123–180°	0·45	151	45·0	17·6	2·56
4	>180°	0·1	—	—	—	—

typical resorptions on a daily dose of 0·1 mg. of fraction 2 make us fear, however, that the material was not as potent as our interpretation of the spectroscopic data had led us to expect.

Discussion.

Most of the work reported in this paper must be regarded as of a preliminary nature and at this stage it is impossible to consider the results from the standpoint of the chemical identity of vitamin E. If we were certain that the intensity of selective absorption at 294 $m\mu$ were a measure of vitamin E potency the problem would be greatly facilitated, but unfortunately at the moment there are discrepancies which make us hesitate to conclude that such is the case. In general we have found that biological potency and absorption at 294 $m\mu$ run parallel during the fractionation, and the results with irradiation and hydrogenation can be reconciled with the view that the vitamin is a substance showing selective absorption at this region of the ultra-violet.

There are, however, other observations, such, for example, as the apparently low biological activity of a distilled fraction showing relatively high intensity of absorption, which make us cautious. The difficulties are in many respects similar to those which were encountered in the early days of the investigation of vitamin A and the most serious one is the absence of any criteria of purity. The information we have obtained does not enable us to say whether our most highly active fractions represent nearly pure vitamin E or whether they are composed of a biologically inert substance in a state of comparative purity contaminated with a small amount of highly active vitamin.

We are inclined to think that our most potent fractions consist to a large extent of one substance. It is true that the evidence from fractional distillation experiments does not support this view, but the amounts subjected to fractionation by this method were so small that too much weight must not be attached to the results.

The analytical data recorded for this active fraction must also be accepted with reserve, until more definite information is forthcoming to show whether or not the material is reasonably homogeneous.

Our studies provide further confirmation of the relative stability of vitamin E. Of its chemical nature we can say little beyond expressing an opinion that it may prove to possess a polycyclic nucleus. We are inclined to dismiss the suggestion put forward by Euler *et al.* [1933] that it may be related to xanthophyll. The vitamin is probably a colourless substance or one showing very slight absorption in the visual spectrum.

Summary.

1. An examination of the unsaponifiable fraction of wheat germ oil has been made with the object of throwing light on the chemical nature of vitamin E.

2. The following substances have been isolated from or detected in this material: sitosterol, dihydrositosterol, ergosterol, dihydroergosterol, lutein, kryptoxanthin, β-amyrin, squalene, a second hydrocarbon of the squalene type,

possibly identical with that found by Channon and Marrian in mammalian livers, a new liquid hydrocarbon which may have the composition $C_{18}H_{32}$ and a lipochrome which may be γ-carotene or rubixanthin. None of these substances appears to be related directly to the vitamin activity.

3. Fractional adsorption on aluminium oxide was found to effect a considerable concentration of vitamin E. The most potent fraction obtained was physiologically active in rat tests in doses of 0·1 mg. daily.

4. The most active fractions were pale yellow and somewhat viscous oils. In the absence of any criteria of purity it is worth recording that analyses of several such fractions show reasonably good agreement, the analytical data indicating a polycyclic structure possibly related to the sterols or amyrins. Two oxygen atoms are present in a molecule of mol. wt. about 440. One of these forms an acetyl derivative but the spectroscopic evidence suggests that the reacting grouping may be keto-enol. If so, the second oxygen is present in a form which does not react with acetic anhydride. The reactions with iodine and hydrogen suggest that three reacting double bonds are present. Whether these observations concern the vitamin itself or another substance which forms the greater part of the active fractions is as yet unknown.

5. The active fractions show a well-marked absorption band with a maximum at 294 $m\mu$ and a minimum at 267 $m\mu$. So far as this investigation has progressed it has shown that the vitamin activity tends to run parallel with the intensity of this band or, more particularly, with its "persistence". It is not yet finally determined whether the band is a characteristic of the vitamin or of an associated substance, but it is significant that with ultra-violet irradiation the reduction in "persistence" is accompanied by loss of biological activity, whereas treatment with hydrogen and palladium, which effects almost complete saturation of the material of the active fraction, does not appreciably lower either the "persistence" of the band or the value as a source of vitamin E.

6. The absorption band at 294 $m\mu$ disappears on acetylation but is shown again by the hydrolysed acetate. As other investigators have noted, biological activity is unaffected by acetylation. The position of the absorption band and its disappearance on acetylation are compatible with the substance containing a ketonic grouping capable of exhibiting tautomeric change.

7. Attempts were made to separate the ketonic substances present in the active fractions in the form of water-soluble mercury complexes. Small yields of "ketonic" material were obtained. These showed no concentration of the substance responsible for the 294 $m\mu$ band.

The authors wish gratefully to acknowledge a grant from the Medical Research Council from which the greater part of the cost of this investigation was defrayed. Messrs Imperial Chemical Industries are also thanked for a personal grant to one of us (E. S.). Valuable assistance was also given by Messrs Vitamins, Ltd., who provided a considerable supply of appropriate wheat germ (Bemax) as well as a contribution towards the general expenses.

It is desired, furthermore, to record appreciation of the help of Mr L. B. Holt, who extracted for us considerable quantities of wheat germ oil and prepared the unsaponifiable fraction.

REFERENCES.

Anderson and Nabenhauer (1924, 1). *J. Amer. Chem. Soc.* **46**, 1717.

—— —— (1924, 2). *J. Amer. Chem. Soc.* **46**, 1957.

—— and Shriner (1926–27). *J. Biol. Chem.* **71**, 395.

Askew (1935). *Biochem. J.* **29**, 472.

Beall and Marrian (1934). *J. Soc. Chem. Ind.* **53**, 309 T.

Bowden and Moore (1933, 1). *Nature,* **131**, 512.

—— —— (1933, 2). *Nature,* **132**, 204.

Burch (1929). *Proc. Roy. Soc. Lond.* A **123**, 271.

Channon, Devine and Loach (1934). *Biochem. J.* **28**, 2012.

—— and Marrian (1926). *Biochem. J.* **20**, 409.

Drummond, Singer and Macwalter (1934). *Chem. Ind.* **53**, 873.

Edisbury (1933). Dissertation, Liverpool University.

Euler, Hellström and Klussmann (1933). *Sven. Kem. Tid.* **45**, 132.

Evans and Burr (1927, 1). *Memoirs Univ. Calif.* No. 8.

—— —— (1927, 2). *J. Amer. Med. Assoc.* **89**, 1587.

Girard (1933). *Bull. Soc. Chim. Biol.* **15**, 562.

Kuhn and Grundmann (1933). *Ber. deutsch. chem. Ges.* **66**, 1746.

Martin, Moore, Schmidt and Bowden (1934). *Nature,* **134**, 214.

Morton and Edisbury (1933). *Nature,* **131**, 618.

Olcott (1934, 1). *J. Biol. Chem.* **105**, *Proc.* lxv.

—— (1934, 2). *J. Biol. Chem.* **107**, 471.

—— and Mattill (1934). *J. Biol. Chem.* **104**, 423.

Thorbjarnarson and Drummond (1934). *Analyst,* **60**, 23.

LIII. SURFACE FILMS OF VITAMIN E CONCENTRATES.

By FREDERIC ANDERTON ASKEW.

From the Sir William Ramsay Laboratories of Inorganic and Physical Chemistry, University College, London.

(*Received December 31st, 1934.*)

CERTAIN preparations described in the preceding paper [Drummond *et al.*, 1935] were examined by the method of surface films, with the object of obtaining information about their molecular structure. The substances examined included an oil concentrate rich in vitamin E, and the crystalline substance M.P. 190° (from acetate M.P. 236°, from E-active fractions 3 and 4).

The substances were spread on the surface of $N/50$ HCl and the surface pressures were measured by the method of Adam and Jessop [1926; Adam, 1930] and the surface potentials by the method of Adam and Harding [1933].

Vitamin E-active oil.

This substance, spread from benzene solution, gave the curve shown in Fig. 1, in which surface pressures are plotted as ordinates and values of area/molecule in sq. Å. as abscissae. The latter were calculated on the assumption that the solute spread consisted of a substance with mol. wt. = 390. Fig. 1 also shows the relation between surface potentials and area, and values of μ calculated from the equation

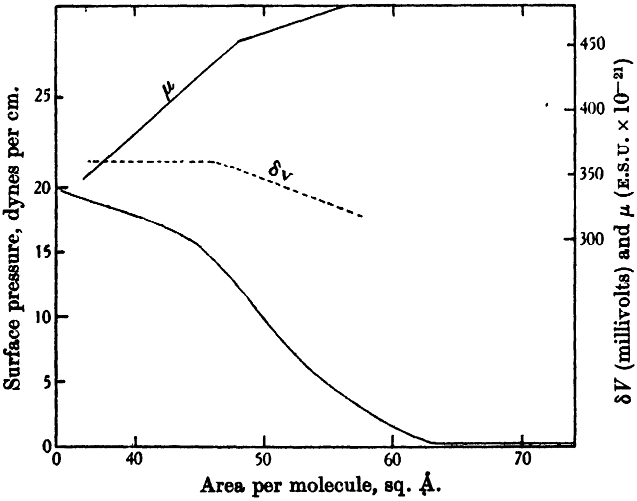

Fig. 1.

$$\delta V = 4\pi n\mu,$$

n being the number of molecules per sq. cm. μ measures the effective vertical component of the dipole moment of the molecule at the corresponding compression.

The surface pressure-area curves are consistent with the view that the substance is polycyclic, with a water-attracting group at or near one end of the molecule. The relatively high compressibility of the film and the limiting area of 63 sq. Å. are similar to those of ketones of the sterol series (*cf.* cholestenone [Rosenheim and Adam, 1929]). If more than one polar group is present in the molecule, it is unlikely that they are far apart in the molecule. If this were so, one would expect the molecule to lie flat on the surface and give a film of the gaseous type (*cf.* pregnandiol, pregnandione, oestrone derivatives [Danielli *et al.*, 1933]).

The films of this substance collapsed when the surface pressure was greater than about 15 dynes/cm.; this collapse was reversible and examination of the films under the ultra-microscope by the method of Zocher and Stiebel [1930] showed the appearance of numbers of fine droplets at about this pressure. The films were unusual in that the reversible collapse was accompanied by a rise in surface pressure; this might be due to the presence of a less readily collapsing impurity. The possibility that the upper flattened portion of the curve was due to a transition between liquid expanded and condensed states was further excluded by measuring the surface pressure at a low temperature (1·5°), which resulted in a curve similar to the one obtained at room temperature (15°).

The moderate changes of surface potential and μ over the unimolecular portion of the film (below 15 dynes/cm.) indicate that only small alterations in the tilt of the molecule and the adhesion of the polar groups to the water took place on compression; beyond this point, where collapse occurred, μ appeared to fall more rapidly, but this was probably due to the area assumed in calculating μ being too small, owing to collapse.

Crystalline substance; M.P. 190°.

This substance on the surface of $N/50$ HCl gave the rather compressible solid film shown in Fig. 2, curve I. Extrapolation to zero pressure gave a limiting area of 47·6 sq. Å. On first compression the film contracted, but above 3 dynes/cm. pressure it formed a stable solid film. The values of surface potential and μ are also shown in Fig. 2.

The data suggest a polycyclic structure with the water-attracting group near one end of the molecule. The limiting area is much in excess of that required for long-chain compounds or terminal groups such as phenol or hexahydrophenol; on the other hand reduced polycyclic structures such as sterols give curves showing limiting areas ranging from 38 sq. Å. upwards, according to the position of the water-attracting group. Among compounds of this type, cholestane-6-ol shows an area of 48 sq. Å. and its surface pressure curve is similar to that of the crystalline substance obtained from vitamin E preparations. The surface potential is somewhat lower.

The empirical formula ($C_{30}H_{50}O$) for the crystalline substance is the same as that of the amyrin series of compounds. These substances are known to be polycyclic, probably containing five rings, and have a hydroxyl group in a position not yet definitely ascertained [Ruzicka *et al.*, 1929; Spring, 1933]. As has been shown in the preceding paper [Drummond *et al.*, 1935] the melting-points of the crystalline vitamin E derivative and its acetate closely correspond with those of β-amyrin. Accordingly, the surface properties of a specimen of β-amyrin were investigated. The results are shown in Fig. 2. On first compression the film showed marked contraction; this effect was similar to that observed with the crystalline substance itself, but occurred to a much greater extent. The initial course which the curve tended to follow is shown as curve III A in Fig. 2. The film rapidly rearranged to give a stable solid film (curve III), rising fairly steeply and tending to a limiting area at zero compression of 47 sq. Å. This initial contraction and rearrangement to give a solid film is common to several substances of the amyrin group (Askew; unpublished measurements).

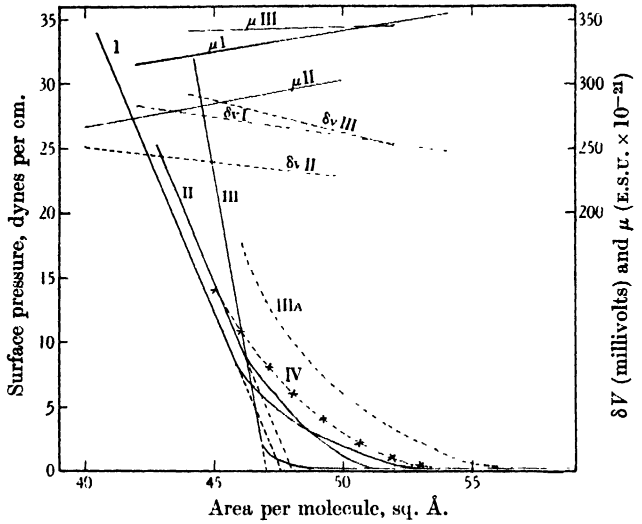

Fig. 2. I. Crystalline substance. II. Cholestane-6-ol. III. β-Amyrin (contracted film). III A. β-Amyrin (initial film). IV. Combined curve, 80 % β-amyrin, 20 % vit. E oil.

The surface pressures of the two substances, although they approach approximately the same limiting areas, differ in two respects: (a) that of β-amyrin is much steeper, (b) it shows the rearrangement phenomena to a much more marked extent. In view of the similarity of the other properties of these two substances and the similarity of their limiting areas, experiments were performed to see whether these differences could be accounted for by impurities in the specimen of the crystalline substance. A mixture of 80 molecules % of β-amyrin and 20 molecules % of the vitamin E-rich oil was spread on the same surface. The resulting curve is shown as curve IV, Fig. 2. The tendency to form a contracting film was greatly diminished, and the slope of the curve approximated to that of the crystalline substance. Hence it appears that the properties of these substances may be greatly modified by the presence of impurities, and a more complete check on the identity of the crystalline substance with β-amyrin awaits the preparation of larger quantities of purified material. At present it can be stated that the substance almost certainly has a polycyclic structure, akin to that of the sterols, with a polar group, most probably a hydroxyl group, near one end of the molecule; that it closely resembles in some of its surface properties substances of the amyrin type, and that the surface data are consistent with the view that the substance is β-amyrin. The surface potentials of the crystalline substance and β-amyrin were of the same order of magnitude, but that of the latter appeared to rise more steeply; this is however uncertain, owing to the tendency of the film to rearrange in the initial stages of compression. The rearrangement was not accompanied by large changes in surface potential.

SUMMARY.

A concentrate rich in vitamin E and a crystalline substance derived from vitamin E preparations have been examined by the method of surface films.

Both substances appear to have condensed polycyclic structures.

The possibility that the crystalline substance is identical with β-amyrin is discussed in the light of surface film data.

The author wishes to express his thanks to Prof. J. C. Drummond for specimens of the substances investigated, to Dr N. K. Adam for much helpful advice and to the Medical Research Council for a maintenance grant.

REFERENCES.

Adam (1930). The physics and chemistry of surfaces. (Oxford Univ. Press.)
—— and Harding (1933). *Trans. Farad. Soc.* **29**, 837.
—— and Jessop (1926). *Proc. Roy. Soc. Lond.* A **110**, 423.
Danielli, Marrian and Haslewood (1933). *Biochem. J.* **27**, 311.
Drummond, Singer and Macwalter (1935). *Biochem. J.* **29**, 456.
Rosenheim and Adam (1929). *Proc. Roy. Soc. Lond.* A **125**, 25.
Ruzicka, Huyser, Pfeiffer and Seidel (1929). *Liebig's Ann.* **471**, 21.
Spring (1933). *J. Chem. Soc.* 1345.
Zocher and Stiebel (1930). *Z. physikal. Chem.* **147** A, 401.

LIV. COPPER METABOLISM IN MAN.

By TUNG-PI CHOU and WILLIAM H. ADOLPH.

From the Department of Chemistry, Yenching University, Peiping, China.

(*Received December 17th, 1934.*)

Recent experiments have shown the importance of copper in haemoglobin formation in the animal body, and the wide distribution of copper in plant and animal material has been repeatedly confirmed. There is a lack of quantitative data however on copper metabolism. We have conducted balance experiments with the object of estimating the copper requirement of man, and in addition we have undertaken copper analyses of human cadavers for the purpose of estimating the total copper content of the human body.

Copper metabolism in normal subjects.

Copper balance experiments were carried out during the late spring and early summer on three different subjects, all Chinese, who consumed simple diets of measured copper content. The copper content of Chinese food materials has been reported elsewhere by Adolph and Chou [1933]. White flour (steamed bread) and milk with a small quantity of vegetables were employed for the low copper diet. An increased copper intake was arranged by replacing the milk with increasing amounts of spinach and pork. Copper was determined in food, urine and faeces by the Biazzo method as modified by Elvehjem and Lindow [1928].

Series 1, in which the copper balance was determined on a subject daily for four successive days, was carried out for the purpose of determining how rapidly the body adjusted itself to altered copper intake. Results are shown in Table I. As a result of this preliminary experiment, it was proposed to employ 2- and 3-day metabolism periods in the series following.

In Series 2, three subjects consumed prescribed diets of increasing copper content over four periods. Each period consisted of 4–6 days, the four periods following each other without interval. The metabolism measurements involving the collection of urine and faeces were confined to the last 3 days and in some cases to the last 2 days of each period. Faeces markers were used and the usual precautions in conducting metabolism balance experiments were observed. The iron intake was not controlled.

The urine and faeces were collected directly in glass or porcelain receptacles. The faeces for each collection period were dried over sulphuric acid and ground in a porcelain mortar. For the analyses, 400 ml. samples of urine were evaporated to a volume of 30–40 ml. and then digested with 40 ml. of sulphuric acid in a Kjeldahl flask till colourless. The dried faecal material was treated in a similar manner. The resultant digest was then transferred to a volumetric flask and diluted so that the acid concentration was about 1 %. In carrying out the analyses the copper was separated from iron by precipitation with hydrogen sulphide, with subsequent determination of the copper in the precipitate as outlined by Elvehjem and Lindow [1928]. All analyses were carried out in duplicate.

Table I. *Copper metabolism in man; intake and output averaged in mg. Cu per day.*

Series 1.

Subject	Sex	Age	Weight kg.	Period	No. of experimental days	Cu intake mg.	Cu output (mg.) Urine	Faeces	Total	Balance
A	F	36	45·5	I	1	0·66	0·27	2·08	2·35	-1·69
				II	1	0·51	0·16	2·43	2·59	-2·05
				III	1	1·72	0·23	1·92	2·15	-0·43
				IV	1	2·17	0·27	1·22	1·49	+0·68
Series 2.										
A	F	37	46·5	I	3	0·67	0·21	1·19	1·40	-0·73
				II	3	2·07	0·23	1·67	1·90	+0·17
				III	2	3·40	0·33	4·49	4·80	-1·42
B	M	17	30·2	I	2	1·11	0·20	1·72	1·92	-0·81
				II	3	1·28	0·18	1·81	1·99	-0·71
				III	3	2·63	0·27	1·84	2·11	+0·52
				IV	2	3·69	0·31	6·41	6·72	-3·03
C	F	62	41·2	I	3	1·09	0·15	1·58	1·73	-0·64
				II	3	2·38	0·27	2·14	2·41	-0·03
				III	2	2·85	0·36	2·37	2·73	+0·12
				IV	2	3·39	0·30	2·43	2·73	+0·66

The results, shown in Table I, indicate that equilibrium was reached when the copper intake was about 2 mg. per day.

Copper content of the human body.

Material from five cadavers, all Chinese, two being adults, was obtained from the Department of Pathology of the Peiping Union Medical College, and analyses made of muscle, liver, heart *etc.* In preparing the material for analysis, the tissues were first washed free from blood and the weighed samples then dried in an electric oven at 60° and ashed in an electric furnace at as low a temperature as possible, and the estimation of copper was carried out by the Biazzo method as for food materials. The results are shown in Table II. In Table III, using the data from the two adult cadavers, figures are calculated for the copper content of each tissue, and also for the total copper content of the body. The copper content of blood is estimated at 5 mg. for the entire body.

Table II. *Distribution of copper in tissues of the human body (amounts expressed in mg. per kg. of dry material).*

	Cadaver 1*	Cadaver 2*	Cadaver 3*	Cadavers 4, 5*
Body weight...	5 kg.	5·5 kg.	22·5 kg.	adults
Sex—age ...	F—3 mons.	F—7 mons.	M—12 yrs.	
Heart	111·1	162·0	—	36·4
Liver	207·2	40·8	45·4	60·0
Kidney	262·6	61·0	54·3	48·5
Pancreas	8·1	46·5	12·3	28·0
Spleen	24·4	—	—	19·6
Lungs	48·5	42·4	18·8	17·3
Adrenal	120·4	—	—	—
Muscle	82·3	72·4	12·7	12·6
Bone	—	—	—	6·5

* Cause of death: (1) meningitis, (2) secondary anaemia, (3) nutritional oedema, (4) opium poisoning, (5) aneurism of aortic arch.

Table III. *Estimate of total copper content of adult human body.*

	Copper content of dry tissue mg./kg.*	Average H_2O content of tissue %†	Average total weight of tissue kg.‡	Total copper content mg.
Heart	36·4	79	0·20	1·5
Liver	60·0	70	1·00	18·0
Kidney	48·5	83	0·09	0·7
Pancreas	28·0	75	0·06	0·4
Spleen	19·6	75	0·09	0·4
Lungs	17·3	79	0·80	2·9
Muscle	12·6	76	21·0	63·6
Bone	6·5	30	5·2	23·4
Blood	—	—	—	5·0§
			Total	115·9

* See Table II. † Figures from Oppenheimer, *Handbuch der Biochemie*, 1.

‡ Figures from standard tables reckoned on basis of 50 kg. man. § Estimated.

DISCUSSION.

The copper balance experiments indicate that equilibrium was reached when the copper intake was approximately 2 mg. per day, and we suggest that this figure represents the daily copper requirement in man. A consideration of the copper content of common foodstuffs, which ranges from 2 to 25 mg. per kg. of dry material, indicates that the average mixed diet readily supplies this amount of copper, particularly if it contains a liberal amount of spinach and similar leafy vegetables.

Inasmuch as copper appears to be associated with iron in haemoglobin formation, it is interesting to compare this figure for copper requirement, 2 mg. per day, with the accepted requirement for iron in man, 15 mg. per day [Sherman, 1932], iron and copper standing in the ratio 7 : 1. Iron and copper requirements per day for the white rat have been accurately determined on the basis of haemoglobin recovery experiments by Hart *et al.* [1928] and Waddell *et al.* [1928], who give the figures 0·5 mg. and 0·06 mg. respectively for iron and copper; these represent an iron-copper ratio of 8 : 1. This ratio is almost identical with that above, and suggests that the outstanding rôle of iron and copper in the human body is expressed in the haemopoietic function.

From the copper balance experiments it is evident that in man copper is mainly excreted through the faeces. This was shown by Filehne [1896] to be the case with dogs and cats, and by Flinn and Inouye [1928] and Lindow *et al.* [1929] to hold also for rats. It is important to note that in spite of changes in the copper intake, our figure for urinary copper remains fairly constant, varying between the limits 0·15 and 0·36 mg. (average 0·25 mg.) per day. Rabinowitch [1933] has recently reported values ranging between traces and 0·7 mg. per day for urinary copper in normal man. The excretion of faecal copper is more irregular, and with two of our subjects a further increase in copper intake was accompanied by a pronounced negative balance and by an apparent breakdown in the storage supply.

Our calculated figure, 115·9 mg., for the copper content of the adult body can be regarded only as a rough estimate. If brain, skin, and other tissues are included this figure should be increased, and it is probable that the total copper content is between 100 and 150 mg. The copper stored in the body is found mainly in the liver, muscles and bones. Our results for copper determinations in adult tissues are slightly higher than those reported by Cunningham [1931].

The higher percentage copper content of certain of the tissues of the two infant cadavers studied is rather marked. A reserve supply of copper in the livers of the newborn has been reported by a number of observers, and discussed by Morrison and Nash [1930], Nitzescu [1931], and Cunningham [1931]. It appears that in certain mammals, including human beings, a reserve storage of both iron and copper is provided to the newborn to insure adequate haemoglobin formation. Cadaver 2, a case of anaemia, exhibits a much smaller amount of copper in the liver. A similar observation is recorded by Morrison and Nash [1930].

SUMMARY.

From copper balance experiments on three adult subjects fed with copper-containing foods, it is shown that the copper requirement in man is approximately 2 mg. per day.

The copper excretion in the urine of the normal individual does not vary appreciably with varied copper intake and averages 0·25 mg. per day.

From analyses of tissues, it is estimated that the adult body contains between 100 and 150 mg. of copper.

We wish to thank Dr C. H. Hu of the Department of Pathology of the Peiping Union Medical College for material used for analysis, and also the Department of Biochemistry of the same institution for laboratory facilities during part of the work.

REFERENCES.

Adolph and Chou (1933). *Chinese J. Physiol.* **7**, 185.
Cunningham (1931). *Biochem. J.* **25**, 1267.
Elvehjem and Lindow (1928). *J. Biol. Chem.* **81**, 435.
Filehne (1896). *Deutsch. med. Woch.* **22**, 145.
Flinn and Inouye (1928). *J. Amer. Med. Assoc.* **90**, 1010.
Hart, Steenbock, Waddell and Elvehjem (1928). *J. Biol. Chem.* **77**, 797.
Lindow, Peterson and Steenbock (1929). *J. Biol. Chem.* **84**, 419.
Morrison and Nash (1930). *J. Biol. Chem.* **88**, 479.
Nitzescu (1931). *Compt. Rend. Soc. Biol.* **106**, 1176.
Rabinowitch (1933). *J. Biol. Chem.* **100**, 479.
Sherman (1932). Chemistry of food and nutrition. (Macmillan, New York.)
Waddell, Elvehjem, Steenbock and Hart (1928). *J. Biol. Chem.* **77**, 777.

LV. THE COPPER AND "INORGANIC" IRON CONTENTS OF HUMAN TISSUES.

By SIDNEY LIONEL TOMPSETT.

From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.

(*Received December 27th, 1934.*)

The present paper is concerned with the concentration of copper and "inorganic" iron in various tissues and bones of the human body and also with suitable methods for their determination.

COPPER.

Several writers have determined the distribution of copper in human tissues. Herkel [1930] obtained values of 2·88–12·9 mg. Cu per kg. fresh liver and ranged in descending order of concentration his results may be summarised as follows: liver, kidney, spleen, pancreas and bone. Cunningham [1931] found that the average concentration of copper in three human livers was 24·9 mg. per kg. of dry tissue and ranged in descending order of concentration his results may be summarised as follows: liver, kidney, brain, pancreas and spleen. Others have published analyses of single organs, especially the liver, which agree more or less with the analyses of the above two writers [Schönheimer and Oshima, 1929; Kleinmann and Klinke, 1930; Cherbuliez and Ansbacher, 1930; Gordon and Rabinowitch, 1933].

The majority of distribution studies such as these must almost necessarily be done with pathological subjects. In certain pathological conditions of the liver in the adult, there appears to be a very marked increase in the concentration of copper. This occurs often in both pigmented and non-pigmented cirrhosis of the liver [Schönheimer and Oshima, 1929; Cherbuliez and Ansbacher, 1930; Kleinmann and Klinke, 1930; Herkel, 1930]. Gordon and Rabinowitch [1933] in one case of yellow atrophy of the liver obtained the extraordinarily high value of 179·3 mg. copper per kg. fresh tissue. Herkel [1930] states that in haemochromatosis the concentration of copper is increased in all the organs, with the exception of the kidney and bone. The copper content of foetal organs appears also to be higher than in adults [Kleinmann and Klinke, 1930; Cunningham, 1931; Sheldon and Ramage, 1931]. Sheldon and Ramage [1931] report that five foetal livers examined by them contained 5–7 times the concentration of copper present in adult organs. In a survey such as is intended here, it will be necessary then to exclude foetal organs and organs from cases of liver disease.

The question of bone seems to have been neglected. Of the above-mentioned writers only Herkel [1930] and Sheldon and Ramage [1931] appear to have examined this material. The former, as a result of two analyses, obtained values of 3·7 and 4·03 mg. per kg. fresh material. Sheldon and Ramage state that bone contains only a trace of copper.

The various colorimetric methods for the determination of copper in biological material have been discussed elsewhere [Tompsett, 1934, 2]. With the exception of Sheldon and Ramage, who used a spectrographic method, the abovementioned analyses were obtained by modifications of the Biazzo method. The writer has found the method of Callan and Henderson [1929] as modified by

McFarlane [1932] to be the most sensitive and accurate. In this method sodium diethyldithiocarbamate is added to the solution under test, a yellow complex with copper is formed and this is extracted by shaking with amyl alcohol. The extract is compared in a colorimeter with a standard prepared similarly. This reaction, although independent of p_H, is carried out in alkaline solution in the presence of pyrophosphate to prevent interference by iron.

Methods.

Tissues. The following method is based on that used for blood and reported previously [Tompsett, 1934, 2].

A porcelain pestle and mortar and some broken glass were treated several times with hot dilute hydrochloric acid to render them copper-free. The tissue to be examined was cut up into small pieces and 10 g. ground up with the broken glass. About 40 ml. of 10 % trichloroacetic acid were added and the grinding continued. The supernatant fluid was filtered through an acid-washed filter-paper and the residue washed with 10 % trichloroacetic acid until the requisite volume of filtrate was obtained. Extracts of liver tissue were made up to 100 ml., while extracts of other organs were made up to 50 ml. The final estimation was made directly, using 20 ml. of extract with a standard containing 0·01 mg. copper as described for blood.

The results obtained by this method were compared with those obtained after ashing either with sulphuric and perchloric acids or by ignition in a silica basin. For the ignition method the tissues (10–20 g.) were mixed with 5 g. of copper-free sodium phosphate for reasons that have been stated in a previous paper in connection with the analyses of samples of diet [Tompsett, 1934, 4].

From the figures shown in Table I it will be seen that the copper contents of tissues as determined by the two methods are the same within the limits of experimental error, that is copper may be extracted completely from tissues by trichloroacetic acid and reacts directly with sodium diethyldithiocarbamate.

Table I. *Copper content of fresh tissue.*

(mg. Cu per 1000 g.)

Tissue		A Determined directly in trichloroacetic acid extract	B Determined after ashing tissue
Liver	1	22·24	22·80
	2	6·20	6·16
	3	5·69	5·74
	4	4·94	5·06
	5	5·26	5·10
Kidney	6	3·33	3·52
	7	3·63	3·45
	8	3·01	2·84
Brain	9	6·96	7·14
	10	2·27	2·20
	11	4·57	4·61
Spleen	12	1·40	1·38
	13	1·16	1·16
	14	2·27	2·18
Pancreas	15	4·00	3·79
	16	1·96	2·06
	17	2·22	2·20

Bone. Bone offers special difficulties in the determination of copper, owing to its high content of calcium phosphate. Since the final determination is carried out at alkaline reaction, precipitation of calcium phosphate would be so great as to interfere with the result. The same difficulties are met with in milk, owing to its relatively high concentration of calcium phosphate and low concentration of copper. In the case of faeces, urine and samples of diet, materials containing relatively much lower concentrations of calcium phosphate, copper may be determined directly in solutions of their ash by addition of sodium citrate to prevent precipitation of the phosphates [Tompsett, 1934, 4]. With bone or milk, however, there is no alternative but to make a preliminary separation of copper.

Many authors have separated copper as the sulphide but this is time-consuming and liable to lead to difficulties. The following method has been found to give accurate and rapid separations of copper.

The sample of bone (30–40 g.) was ashed in a silica basin. The final traces of carbon were destroyed by the addition of 10 ml. concentrated nitric acid and further heating. The ash was dissolved in distilled water containing 15 ml. of concentrated hydrochloric acid filtered through an acid-washed filter-paper and diluted to 250 ml. A sample of 50 ml. was measured into a 750 ml. separating funnel and diluted to about 400 ml. with distilled water. To the solution 100 ml. of 20 % sodium citrate were added and the whole made slightly alkaline to litmus by the addition of ammonia. After the addition of 5 ml. of 2 % aqueous sodium diethyldithiocarbamate, the yellow copper complex which was formed was extracted with ether. 25 ml. of ether were added and the whole vigorously shaken. A further 25 ml. of ether were added and the vigorous shaking repeated. The ether layer containing the copper complex separated very quickly. The aqueous layer was run off and the ether washed twice with about 50 ml. of distilled water. The ether extract was then run off into a 300 ml. Kjeldahl flask and the separating funnel rinsed with 25 ml. of ether which was transferred to the Kjeldahl flask. The aqueous layer and washings were re-extracted with 25 ml. ether which was washed and added to the other ether extracts. The ether was evaporated off on a steam-bath and the organic matter destroyed by heating with 1 ml. concentrated sulphuric acid and 1 ml. of perchloric acid. The contents of the flask were diluted to 25 ml. with distilled water and the copper content determined in an aliquot (about 5 ml.) using 0·01 mg. Cu as standard.

From the results shown in Table II it will be seen that copper added to solutions of bone ash could be recovered quantitatively by this technique.

Table II. *The recovery of copper added to an acid solution of bone ash.*

	Initial copper content mg.	Copper added mg.	Total copper content mg.	Copper recovered mg.
1	0·046	0·050	0·095	0·049
2	0·046	0·075	0·118	0·072
3	0·046	0·100	0·152	0·106
4	0·064	0·050	0·111	0·047
5	0·064	0·075	0·144	0·080
6	0·064	0·100	0·157	0·093

Table III gives the copper content of cows' milk as determined by the above procedure. No special precautions were taken in the collection of this milk which was unpasteurised and delivered in iron cans. These figures agree very closely with those obtained in America by Lindow *et al.* [1929] who obtained an average value of 0·15 mg. Cu per litre.

Table III. *The copper content of cows' milk.*

(mg. Cu per litre.)

1	0·11	3	0·14	5	0·11	7	0·10
2	0·13	4	0·12	6	0·16	8	0·14

Average—0·13.

"INORGANIC" IRON.

No figures for the "inorganic" iron content of human tissues appear to be available. Copper may be extracted from biological materials with trichloroacetic acid but "inorganic" iron is extracted either partially or not at all [Tompsett, 1934, 1, 3].

It has been shown that the whole of the iron of egg yolk is in the "inorganic" form, which confirms the earlier work of Hill [1931]. When a suspension of egg yolk was treated with trichloroacetic acid, no iron could be detected in the filtrate, but if, prior to the precipitation of the proteins, thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate were added, the whole of the iron was present in the filtrate. This work was extended further when it was shown that this phenomenon observed with egg yolk was due to the presence of indiffusible phosphorus compounds, *e.g.* phospholipins and phosphoproteins. When solutions of iron salts were added to a suspension of lecithin or solutions of caseinogen or to milk and the protein or phospholipin precipitated with trichloroacetic acid, no iron could be detected in the filtrate, but if thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate were added prior to the addition of trichloroacetic acid, the iron could be estimated quantitatively in the filtrates. On the other hand, when mixtures of ferric salts and either egg white or solutions of edestin were treated with trichloroacetic acid, the iron could be estimated quantitatively in the filtrates. It has been suggested that ferric but not ferrous iron forms complexes with these indiffusible phosphorus compounds, and that upon reduction of the ferric iron by sodium hydrosulphite or thiolacetic acid these complexes are destroyed. The complex also appears to be unstable in the presence of sodium pyrophosphate which is probably due to the property of iron of forming non-ionised compounds with pyrophosphates.

The iron was detected and determined with thiolacetic acid, which gives a purple colour with iron salts on the addition of ammonia. The reaction is very delicate, is quantitative and is not affected by the presence of organic substances such as may be present in filtrates like the above. Both ferric and ferrous salts give the reaction since the former are reduced to the ferrous state by thiolacetic acid.

In his investigations Hill [1931] used $\alpha\alpha'$-dipyridyl. This substance gives a red colour with ferrous salts and no reaction with ferric salts. He found that when egg yolk was suspended in an acid acetate buffer and $\alpha\alpha'$-dipyridyl added, no colour developed, whereas when a reducing substance such as sodium hydrosulphite was added, a red colour developed. When this colour was compared with standards, he noted that it accounted for all the iron of the yolk.

McFarlane [1934] has studied the iron of the rat's liver. He found that trichloroacetic acid extracts gave only faint reactions for iron with the thiocyanate reagent, whereas the "inorganic" iron as determined by Hill's $\alpha\alpha'$-dipyridyl method was very much higher.

Method.

For the complete extraction of "inorganic" iron by trichloroacetic acid, the complexes must be broken down first. For this type of extraction it was considered that sodium pyrophosphate would answer this purpose better than a reducing agent such as thiolacetic acid or sodium hydrosulphite. The extraction and estimation were carried out as follows:

Some broken glass and 10 g. of finely cut tissue were ground up in a porcelain mortar. 20 ml. of 4 % sodium pyrophosphate were added and the grinding continued. The mixture was allowed to stand 15 minutes after which 20 ml. of 20 % trichloroacetic acid were added and the mixture ground. After a further 15 minutes the mixture was filtered through an acid-washed filter-paper and the residue washed with 10 % trichloroacetic acid until the volume of the extract was either 100 ml. (liver and spleen) or 50 ml. (other tissues). An aliquot portion of the filtrate, containing no more than 0·03 mg. Fe, was diluted to 5 ml. with distilled water and 6 drops of thiolacetic acid were added, followed by 1 ml. of ammonia (sp. gr. 0·88). This was compared in a colorimeter with a standard similarly prepared and containing 0·005, 0·010 or 0·020 mg. Fe.

If copper is being estimated in the same tissue as "inorganic" iron, it may be determined in the extract prepared for the determination of "inorganic" iron, so that only one extraction is necessary.

Table IV.

A. *Copper.*

The figures are expressed in mg. Cu per kg. fresh tissue (1) and in mg. Cu per organ (2).

	Liver		Kidney		Brain		Spleen		Pancreas	Vertebra	Rib
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(1)	(1)
1	3·09	3·40	2·27	0·54	—	—	—	—	2·10	2·83	6·40
2	9·09	12·99	2·36	0·83	2·27	3·18	—	—	1·96	1·63	32·06
3	6·59	15·82	3·82	1·91	4·57	6·40	—	—	2·20	2·84	8·95
4	5·43	8·14	3·57	1·25	3·16	4·42	—	—	—	2·13	47·70
5	4·86	8·75	2·56	0·82	—	—	1·16	0·20	2·46	4·88	10·21
6	3·74	5·24	2·12	0·44	—	—	2·27	0·36	2·36	3·40	3·71
7	2·96	3·26	—	—	2·16	2·81	1·96	0·28	2·06	1·81	4·02
8	5·46	9·83	2·91	1·02	3·96	5·44	2·04	0·28	2·22	2·96	9·81
9	7·94	11·81	3·42	1·02	4·84	6·54	1·96	0·20	2·86	3·04	21·62
10	3·16	5·21	2·16	0·69	2·22	2·84	1·84	0·20	2·54	2·91	14·61
11	4·44	6·04	2·84	1·11	3·04	3·65	2·41	0·28	2·16	1·84	9·45
12	6·12	11·97	3·01	0·84	3·24	3·56	1·92	0·23	2·04	4·16	8·61

B. *"Inorganic" iron.*

The figures are expressed in mg. Fe per kg. fresh tissue (1) and in mg. Fe per organ (2).

	Liver		Kidney		Brain		Spleen		Pancreas	Vertebra	Rib
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(1)	(1)
1	162·4	178·6	10·1	2·4	—	—	93·6	9·4	18·2	128·6	103·4
2	36·6	51·2	9·6	3·4	6·2	8·7	—	—	10·8	123·1	147·6
3	69·2	166·0	3·3	1·6	14·8	20·7	—	—	7·0	142·9	119·5
4	50·6	75·9	9·2	3·0	7·6	10·6	—	—	—	134·0	111·8
5	80·7	144·3	5·1	1·6	4·0	5·2	169·4	29·6	4·2	133·2	114·6
6	27·7	38·8	7·4	1·6	—	—	100·3	10·0	13·4	167·4	161·4
7	64·6	71·1	7·9	1·5	8·6	11·2	96·4	14·5	8·4	146·1	151·8
8	71·4	128·5	4·6	1·4	11·1	15·5	164·6	23·0	6·7	126·0	111·0
9	45·6	68·4	5·4	1·6	14·6	19·7	84·5	8·9	8·4	111·6	109·2
10	84·2	138·9	8·4	2·7	8·4	10·8	126·3	13·9	9·3	136·4	146·1
11	91·6	124·6	7·6	3·0	11·2	13·4	116·4	15·1	8·4	154·3	138·4
12	39·4	76·4	6·9	1·9	6·6	7·3	84·6	10·2	7·1	121·4	109·6

THE COPPER AND "INORGANIC" IRON CONTENTS OF TISSUES.

In Table IV are shown the concentrations of copper and "inorganic" iron in the tissues of 12 cases. These cases had no organic disease of the liver. The bones examined were the rib and the vertebra. Figures are given for the iron content of these bones. These were determined in the solutions of their ashes by the thiolacetic acid method as used for the tissue extracts. Although these figures cannot be classed as "inorganic" iron yet it is more than likely that the greater part of the iron in bones is in the inorganic form. A precipitate of calcium phosphate always appears during the estimation of iron in bone but this may be removed by centrifuging without interfering with the results.

DISCUSSION.

In a previous paper [1934, 2] the writer has reported that when the proteins of blood are precipitated with trichloroacetic acid, the whole of the copper is present in the filtrate and reacts directly with sodium diethyldithiocarbamate. It has been shown in this paper that the copper of tissues such as liver, kidney, brain, pancreas and spleen may be completely extracted with trichloroacetic acid and the copper may be determined directly in the extracts with sodium diethyldithiocarbamate in the same way as blood. During the progress of this work McFarlane [1934] published a paper on similar lines. He extracted rat livers with trichloroacetic acid, found that these extracts gave a direct reaction for copper with sodium diethyldithiocarbamate and that direct estimations using these extracts agreed with those obtained after ashing.

A method has been described for the extraction of copper from solutions of bone and milk ash by means of sodium diethyldithiocarbamate and ether. This extraction is independent of p_H so that it is as efficient in acid as in alkaline solution. It is carried out in alkaline solution in the presence of pyrophosphate to prevent the extraction of iron. If the extraction be carried out as soon as the solution of the ash is obtained it is not necessary to add pyrophosphate since sufficient of this substance is formed during the ignition process. Since the reagent itself is insoluble in ether, the ethereal extracts contain only minimum amounts of organic material. Chloroform was tried as a solvent but it was found that the solubility of the copper complex in this solvent is very poor, nor does chloroform separate very easily.

The values obtained for the copper content of various tissues with the exception of bone agree with those obtained by other workers. Vertebra contains a very low concentration of copper which is fairly constant throughout the series. Rib on the other hand appears to contain very varied amounts of copper, exceeding that in the liver in many cases even when differences of water concentration are taken into consideration. It appears possible that the bones may act as stores for copper as well as the liver.

As would be expected, liver and spleen contain the highest concentrations of "inorganic" iron. Tissues such as brain, kidney or pancreas contain on an average less than a tenth of the concentration of "inorganic" iron of the liver or spleen. The values obtained for the spleen are more constant than those for the liver but from the size of the latter organ it must be the principal store of "inorganic" iron. Vertebra and rib contain fairly high concentrations of iron and, in contrast to copper, the concentrations are almost identical in the two types of bone.

The term "inorganic" iron is probably incorrect since this iron will be in some form of organic combination. It may be justified since ferric salts added to egg yolk *etc.* react in the same way and require the same methods for their separation.

All tissues such as have been examined contain considerable amounts of haematin iron. It would appear, however, that the whole or almost the whole of the iron present in plasma is in the "inorganic" form. The writer [1934, 1] has estimated the "inorganic" iron content of normal sera and obtained values of 0·12–0·22 mg. per 100 ml. Fowweather [1934] in a recent paper has estimated the total iron of plasma, obtaining values of 0·06–0·18 mg. per 100 ml. He noted that precipitation of the proteins with trichloroacetic acid removed a considerable portion of the iron.

SUMMARY.

1. Copper may be determined directly with sodium diethyldithiocarbamate in trichloroacetic extracts of liver, kidney, spleen, brain and pancreas.
2. A method has been described for the determination of copper in bone and milk.
3. The copper and "inorganic" iron contents of a series of tissues including bone have been determined.
4. The values obtained for the copper contents of liver, spleen, kidney, pancreas and brain agree with those obtained by other writers. Vertebra contains very low concentrations of copper while the concentrations in rib are extremely variable.
5. Liver and spleen contain high concentrations of "inorganic" iron whereas kidney, brain and pancreas contain very low concentrations.

In conclusion I wish to thank Dr A. B. Anderson for his helpful criticism and advice and the Medical Research Council for a grant towards expenses.

REFERENCES.

Callan and Henderson (1929). *Analyst*, **54**, 650.
Cherbuliez and Ansbacher (1930). *Arch. klin. Med.* **278**, 365.
Cunningham (1931). *Biochem. J.* **25**, 1267.
Fowweather (1934). *Biochem. J.* **28**, 1160.
Gordon and Rabinowitch (1933). *Arch. Int. Med.* **51**, 143.
Herkel (1930). *Beitr. Path. Anat.* **85**, 513.
Hill (1931). *Proc. Roy. Soc. Lond.* B **107**, 205.
Kleinmann and Klinke (1930). *Arch. Path. Anat. Physiol.* **275**, 422.
Lindow, Elvehjem and Petersen (1929). *J. Biol. Chem.* **82**, 465.
McFarlane (1932). *Biochem. J.* **26**, 1022.
—— (1934). *J. Biol. Chem.* **106**, 245.
Sheldon and Ramage (1931). *Biochem. J.* **25**, 1608.
Schönheimer and Oshima (1929). *Z. physiol. Chem.* **180**, 249.
Tompsett (1934, 1). *Biochem. J.* **28**, 1536.
—— (1934, 2). *Biochem. J.* **28**, 1544.
—— (1934, 3). *Biochem. J.* **28**, 1802.
—— (1934, 4). *Biochem. J.* **28**, 2088.

LVI. THE REDUCTION OF DINITROPHENOLS BY REDOX INDICATORS AND ENZYMES.

BY GUY DRUMMOND GREVILLE[1] AND KURT GUENTER STERN.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

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Two types of reagent are known to cause an increase in the respiration of surviving tissues and yeasts when added to the medium in which these cells are respiring: firstly certain organic dyestuffs which form reversible and electro-active systems with their readily autoxidisable leuco-forms [Barron, 1930; Chang and Gerard, 1931; Gerard, 1932; Friedheim, 1932; 1934; Fleischmann and Kann, 1933; Dickens, 1934]; and secondly the dinitrophenols [Dodds and Greville, 1933; 1934; Ehrenfest and Ronzoni, 1933; Field *et al.*, 1933; 1934].

As in many ways there is a striking resemblance between the actions of the two types of agent, which even extends to their effects on the anaerobic glycolysis of frog muscle [Gerard, 1931; Ehrenfest and Ronzoni, 1933], it seemed of interest to inquire whether the dinitrophenols can be found in any way to form a reversible system under physiological conditions. A nitrophenol might conceivably form an oxidation-reduction system in two ways: it might be the oxidant of a system the reductant of which is formed by reduction of a nitro-group, or it might be the reductant of a system the oxidant of which is formed by oxidation of the phenolic hydroxyl group. From the data of Fieser [1930, 1, 2] it would appear that the oxidation of a nitrophenol would require an oxidation intensity far in excess of that possessed by living cells or by any system so far isolated from them (about 1 v.). Hence for the present problem the second alternative may be neglected. On the other hand it is known that dinitrophenol is reduced by the living animal to nitroaminophenols [Guerbet and Mayer, 1932]. We have therefore concentrated on the reduction of nitrophenols by various agents. An attempt was made to see whether this would lead to the formation of a reversible system, and whether the reduction could be performed by enzymes extractable from living cells.

EXPERIMENTAL.

Tests for reversibility.

Potentiometric experiments. We tested for reversibility at p_H 7 by the methods commonly employed in potentiometric analysis, namely the methods of reductive and oxidative titration and the method of mixtures.

Titration of $10^{-3} M$ 4:6-dinitro-*o*-cresol with hydrosulphite did not yield typically S-shaped curves. The potentials measured on the two electrodes employed were not stable and differed widely. Flattening of the curves in different experiments occurred in different E_h ranges. The colour changes observed during the course of the titration were: lemon-yellow (before addition of the titrant) → orange → red → colourless.

The same sequence of colour changes could be observed on reduction with hydrogen in the presence of colloidal palladium. Since reversibility of the system was the more likely to be found the less extensive the reduction, the dinitrocresol was reduced by palladium-hydrogen under photo-electric control to maximum red colour production, presumably to the nitroaminocresol stage. The hydrogen was then replaced by nitrogen and the system was titrated back with ferricyanide. Each addition of a small amount of the titrant caused an initial rise of the potential followed by a drifting back to approximately the original value, until finally a point was reached where the potential suddenly rose into the potential range of the ferricyanide system and remained there. During the titration the colour changed from dark red to dark brown.

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[1] Halley-Stewart Research Fellow.

Finally the method of mixtures was tried. An equimolar amount of 4-nitro-2-aminophenol was added to a de-aerated buffered solution of 2:4-dinitrophenol. No steady potential was reached within 40 mins. and the two electrodes disagreed. A small quantity of methylene blue was added without any stabilising effect being observed. In order to see whether the presence of living cells would cause the system to attain a stable potential, a small quantity of washed baker's yeast was added to an equimolar mixture of dinitrophenol and nitroaminophenol. This caused the potential to become somewhat more negative. When methylene blue was added to the system, a slow potential decrease was observed with a flattening of the time-potential curve as the poising range of the indicator was approached. It follows that the presence of the nitrophenols did not alter the course of events which normally occurs with de-aerated suspensions of yeast cells in the presence of methylene blue [Cannan *et al.*, 1926].

Experiments with mammalian erythrocytes.

It has been found that reversible oxidation-reduction systems, the normal potentials of which may vary within very wide limits, cause an increase in the oxygen uptake of non-nucleated red cells, the respiration of which is normally very low [Harrop and Barron, 1928; Michaelis and Salomon, 1931; De Meio *et al.*, 1934]. Hence if nitrophenols were found to increase catalytically the oxygen uptake of such cells, this would provide evidence for their conversion into a reversible system when in contact with living matter. Experiments with human and rabbit erythrocytes, however, failed to demonstrate any such effect. The results of a typical experiment are given below.

Material: Washed rabbit erythrocytes made up to blood volume with phosphate-saline mixture, containing 0·25 % lithium *d*-lactate, p_H 7·3.

Each Warburg vessel contained 2 ml. of this suspension, oxygen capacity 142 μl.

Temperature: 38°. Observation time: 60 mins.

Added agent	O_2 uptake in μl.
None	2·5
$10^{-4}M$ methylene blue	112
$10^{-4}M$ thionine	115
$3.10^{-6}M$ thionine	35
$10^{-3}M$ dinitro-*o*-cresol	2
$10^{-4}M$ dinitro-*o*-cresol	3
$10^{-5}M$ dinitro-*o*-cresol	1
$3.10^{-6}M$ dinitro-*o*-cresol	3·5
$10^{-6}M$ dinitro-*o*-cresol	0

In contrast to these findings the respiration of avian (nucleated) erythrocytes, which normally is already considerable, is greatly increased by dinitrophenol [De Meio and Barron, 1934]. Dinitrophenols have so far been shown to exert their effect only on those cells which already possess a complete respiratory system.

REDUCTION EXPERIMENTS.

*Reaction of dinitro-*o*-cresol with redox indicators.*

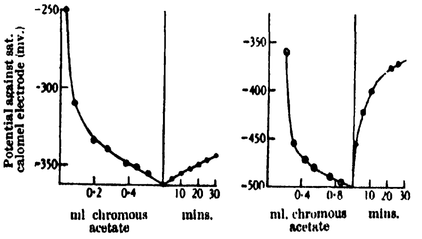
We first sought to determine the level of reduction intensity at which, in the absence of bio-catalysts, the rate of reduction of the nitro-groups in dinitro-*o*-cresol becomes appreciable. A scale of reduction intensities is provided by a series of partially reduced redox indicators. The method employed was that of "potentiometric indicators" [Conant and Lutz, 1923], which consists in determining electrometrically the rate of change of potential following the addition of the substance under investigation to equilibrated mixtures of redox indicators and their leuco forms. For discussion of the theory of the method the reader is referred to Fieser [1930, 2].

Seven indicator systems were employed, covering the E_h range between +0·040 and −0·270 v. All experiments were carried out at p_H 7·3 and at a temperature of 30°. The apparatus used is described below. 40 ml. of a buffered solution of the selected indicator were titrated with chromous acetate to a definite potential well inside the poising range. In most cases a value near the normal potential (E_0') of the indicator was selected. When the electrode potential had become perfectly steady, an equimolar amount of dinitro-*o*-cresol dissolved in 1 ml. of carefully de-aerated buffer was added. The total concentration of the indicator was $10^{-4} M$. In addition one experiment with gallophenin was carried out with a five times higher concentration of dyestuff and dinitrocresol. The results are shown in Table I, and some curves are given in Fig. 1.

Table I.

Redox indicator	E_0' at p_H 7·3 v.	Increase of potential in mv. after 15 mins.	30 mins.	60 mins.	90 mins.	E_h t=0 mins.	t=30 mins.	% oxidation t=0 mins.	t=30 mins.	Increase %
Thionine	+0·040	0	0	0	0	+0·041	+0·040	50	50	0
Indigotetrasulphonate	−0·059	0	0	0	0	−0·056	−0·055	55	55	0
Indigotrisulphonate	−0·094	4	3	3	3	−0·0875	−0·0845	62	67·5	5·5
Indigodisulphonate	−0·138	4	11	—	—	−0·118	−0·107	84	91·5	7·5
Indigodisulphonate	−0·138	12·5	21	36	—	−0·130	−0·109	65	91	26
Gallophenin	−0·157	1	1	2	1	−0·157	−0·156	50	50	0
		4	7	14	16	−0·158	−0·1505	50	62	12*
1:5-Anthraquinone-disulphonate	−0·220	26	35	—	—	−0·197	−0·162	85·5	94	8·5
Anthraquinone-β-sulphonate	−0·270	114	136	—	—	−0·267	−0·1309	55·5	100	44·5

* Five times higher concentrations.



Indigodisulphonate

Anthraquinone-β-sulphonate

Fig. 1.

It is obvious that the rate of reduction of dinitrocresol is not determined solely by the potential of the indicator system, which finding is in agreement with the observations of Conant and Lutz [1923]. It may, however, be said that the rate of reduction is negligible above −0·05 v., small between −0·05 and −0·20 v., and considerable below −0·25 v. It is clear that this statement holds only for the experimental conditions chosen.

Reduction of dinitrophenols by enzyme systems.

It is now known that the activation of different substrates, *e.g.* formate or lactate, by the appropriate dehydrogenase systems gives rise to different levels of reduction intensity. Certain dehydrogenases (succinic, lactic and xanthine dehydrogenases) indeed are found to set up with the oxidised and reduced forms of their substrates reversible systems whose potentials have been measured by indirect methods [for literature see Stern, 1934, 2]. We have studied the reduction of dinitrophenols by four different dehydrogenase-substrate systems whose reduction intensities cover a wide range.

(1) *Formic dehydrogenase.* Though its reversibility has not yet been established, the formic dehydrogenase-formate system reduces very negative indicators so easily that a very high reduction intensity is indicated. One might therefore expect that dinitro-*o*-cresol would be easily reduced by this system. It was nevertheless found that formic dehydrogenase in the presence of formate caused no visible reduction of dinitro-*o*-cresol in the Thunberg tube. If, however, a small quantity of benzyl viologen (E_0' −0·359 v.) was added, a brown colour rapidly appeared. The protocol of a typical experiment is given below.

Formic dehydrogenase prepared by tryptic digestion of a washed suspension of *Bact. coli commune* Escherich according to the method of Stickland [1929]. 0·1 ml. of this preparation reduced 1 ml. of 1/5000 methylene blue in the presence of formate in 3 mins. at 21°. In the absence of substrate the reduction time exceeded 16 hours.

Benzyl viologen was prepared by the method of Michaelis and Hill [1933].

The experiment was carried out in vacuum tubes at 38° in the manner described below.

Solutions: phosphate buffer, 0·1 M, p_H 7·3; dinitro-*o*-cresol, 10^{-2} M; benzyl viologen, 4 . 10^{-3} M; sodium formate, 0·2 M.

Tube	1	2	3	4
	ml.	ml.	ml.	ml.
Buffer	2·0	2·5	2·0	2·0
Enzyme	0·5	0·5	0·5	0·5*
Substrate	—	0·2	0·2	0·2
Dinitrocresol	0·4	0·4	0·4	0·4
Viologen	0·5	—	0·5	0·5
Water	0·6	0·4	0·4	0·4
At beginning:	Lemon-yellow	Lemon-yellow	Lemon-yellow	Lemon-yellow
After 5 mins.:	No change	No change	Brown-yellow	No change
After 45 mins.:	No change	No change	Red-brown	No change

* Heated to 100°.

Passing oxygen through the solution in tube 3 at the end of the experiment for 30 mins. resulted in no change of colour.

Experiments with 2:4-dinitrophenol led to similar results. The coloured reaction product was identified in this case as 4-nitro-2-aminophenol by following the procedures described by Guerbet [1932], who has worked out methods for the separation and detection of small quantities of the reduction products of 2:4-dinitrophenol. 30 ml. of reaction mixture were incubated at 30° for 5·5 hours in a stream of pure nitrogen. At the end of the experiment diaminophenol was tested for with dichromate with a negative result. This test was found to be sensitive to 1/50,000 diaminophenol added to the reaction mixture. 4-Nitro-2-aminophenol was separated in aqueous solution from the remainder of the reaction mixture by Guerbet's procedure and identified by Derrien's reaction (diazotisation and coupling with β-naphthol). The characteristic two-banded spectrum was observed after the addition of zinc acetate in methyl alcohol to the ethereal solution of the azo-β-naphthol. The amount of 4-nitro-2-aminophenol formed was roughly estimated by comparison in Nessler glasses with a standard solution of this compound (solvent: 5 % ammonia). From this comparison the degree of conversion was seen to be at least 45 %. 2-Nitro-4-aminophenol could not be detected.

In order to obtain insight into the mechanism of the coupled reaction observed in the Thunberg tube experiments, the system was subjected to potentiometric and photometric analysis in the apparatus described below. The phenomena observed in these experiments are represented in the composite diagrams of Fig. 2. In the first experiment (upper diagram), after the formic dehydrogenase-formate system had been allowed to reduce the benzyl viologen, the addition of a relatively large amount of dinitro-o-cresol caused a sudden rise in potential to a level which was maintained for over 3 hours. The violet colour of the reduced indicator was discharged immediately after addition of the dinitrocresol, and during the subsequent observation time the solution became

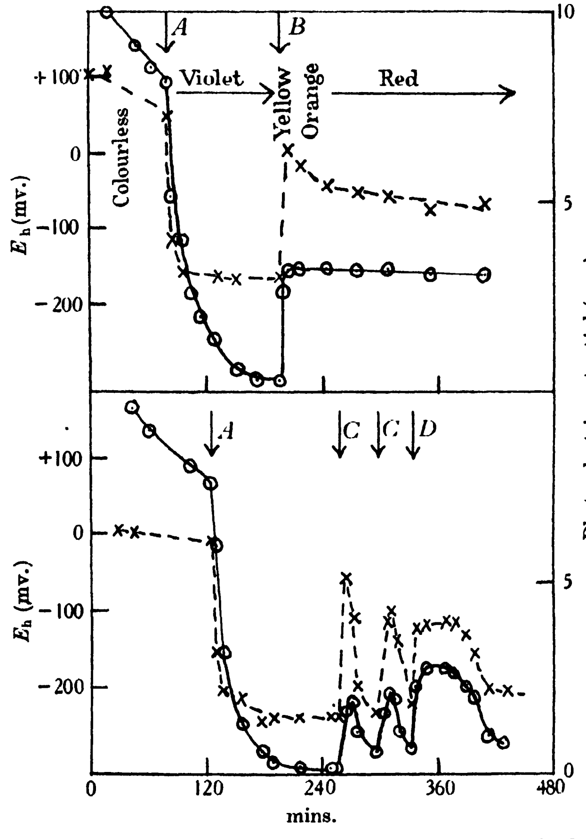


Fig. 2. Formic dehydrogenase and benzyl viologen. *A*, Formate added. *B*, 1 ml. dinitrocresol added. *C*, 0·1 ml. dinitrocresol added. *D*, 0·3 ml. dinitrocresol added. ——— Electrode potentials. - - - - Photo-electric potentials.

increasingly red. At the steady potential level attained no appreciable quantities of the reduced viologen indicator could exist. It was concluded therefore that the enzyme-formate system reduces the viologen, and the reduced form of the indicator reacts with the dinitrocresol (when the latter is in excess) as fast as it is formed. This view was confirmed by the experiment shown in the lower part of Fig. 2, in which successive small quantities of dinitro-*o*-cresol were added. Here the potential fell and the purple colour of reduced viologen appeared each time that the whole of the dinitrocresol present had been used up.

In addition to benzyl viologen the following indicators were found to bring about the coupled reduction of dinitro-*o*-cresol: brilliant cresyl blue ($E_0' + 0{\cdot}045$ v. (p_H 7)), Nile blue ($E_0' - 0{\cdot}116$ v. (p_H 7)), Janus green ($E_0' - 0{\cdot}256$ (p_H 7)). In order to judge the degree of reduction of the dinitrocresol at the end of the experiments the dyestuffs were removed after admission of air by shaking with kieselguhr and filtering.

(2) *Xanthine oxidase.* The redox potential of the hypoxanthine-enzyme-uric acid system is $-0{\cdot}44$ v. at p_H 7 [Filitti, 1933]. It was found that this system, in contrast to the formic system, was able to reduce dinitrocresol in the absence of any indicator. The rate of reduction, however, was increased by the addition of benzyl viologen.

Enzyme: whey preparation of Dixon and Kodama [1926]. 1 ml. of the solution used reduced 1 ml. of 1/5000 methylene blue in the presence of 0·2 mg. of hypoxanthine in 1 min. 50 secs. at 37°.

Solutions: phosphate buffer, $0{\cdot}1\,M$, p_H 7·3; hypoxanthine, 1 mg. per ml.; benzyl viologen, $2.10^{-3}M$; dinitro-*o*-cresol, $10^{-2}M$.

Temperature: 37°.

Tube	1	2	3	4
	ml.	ml.	ml.	ml.
Buffer	1·0	1·0	1·0	1·0
Enzyme	1·0	1·0	1·0	1·0
Substrate	—	0·2	0·2	0·2
Dinitrocresol	0·3	0·3	0·3	—
Viologen	—	—	0·5	0·5
Water	0·7	0·5	—	0·3
At beginning:	Lemon-yellow	Lemon-yellow	Lemon-yellow	Colourless
After 15 mins.:	No change	Slightly orange	Deep orange	Purple
After 1 hour:	No change	Orange	Brown	Purple

2:4-, 2:5-, and 2:6-dinitrophenols are also reduced by this enzyme system. An attempt to identify the brown reduction product of 2:4-dinitrophenol failed.

Since the xanthine oxidase system showed such an interesting difference from the formate system in that no extraneous carrier was required for the reduction of dinitrocresol, we subjected this reduction process to potentiometric and photo-electric analysis (Fig. 3). Whilst reduction of the dinitrocresol was proceeding in the absence of benzyl viologen, as seen from the light transmission curve, the potential remained fairly steady at a comparatively positive level. Addition of benzyl viologen caused the potential to fall. Another experiment conducted in a manner similar to that of the second experiment with formic dehydrogenase (Fig. 2, lower part) gave results similar to that experiment; in both the reduction of dinitrocresol proceeded at approximately the same potential level.

(3) *Lactic dehydrogenase.* The normal potential of the lactate-enzyme-pyruvate system has been found by various workers to be near $-0{\cdot}200$ v. at p_H 7 [see Stern, 1934, 2]. It was found that yeast lactic dehydrogenase in the presence of lactate would not reduce dinitrocresol to a brown compound, but in the

presence of the following indicators it was capable of doing so: pyocyanin (E_0' −0·034 v. (p_{H} 7)), toxoflavin (E_0' −0·049 v. (p_{H} 7); [Stern, 1935], β-anthraquinonesulphonate (E_0' −0·250 v. (p_{H} 7)).

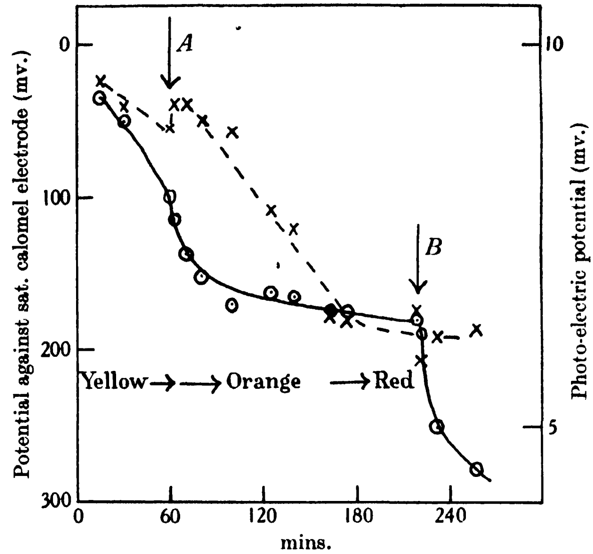


Fig. 3. Xanthine oxidase + dinitro-*o*-cresol, p_{H} 7·3, 30°. *A*. Hypoxanthine added. *B*. Benzyl viologen added. ——— Electrode potentials. – – – – Photo-electric potentials.

Enzyme: extracted from acetone-yeast by the method of Bernheim [1928]. The extract was dialysed against 0·15 *M* phosphate buffer p_{H} 7·3 to minimise volume increase. 0·5 ml. enzyme solution reduced 1 ml. 1/5000 methylene blue in presence of lactate in 14 mins. at 38°.

Solutions: phosphate buffer, p_{H} 7·3, containing enzyme; dinitro-*o*-cresol, $2.10^{-2}M$; lithium *d*-lactate, *M*; pyocyanin (from *Ps. pyocyanea*), $2.10^{-3}M$; toxoflavin (from *Bact. bongkrek*), $2.10^{-3}M$.

Temperature: 38°.

Tube	1	2	3	4	5
	ml.	ml.	ml.	ml.	ml.
Enzyme	2·0	2·0	2·0	2·0	2·0
Substrate	0·5	0·5	0·5	0·5	0·5
Dinitrocresol	0·2	—	0·2	—	0·2
Pyocyanin	—	0·3	0·3	—	—
Toxoflavin	—	—	—	0·5	0·5
Water	0·3	0·2	—	1·0	0·8
At beginning:	Lemon-yellow	Blue	Green	Yellow	Yellow
After 1 hour:	No change	Colourless	Orange	Colourless	Yellow
After 3 hours:	No change	Colourless	Brown-orange	Colourless	Orange

The reaction product in the case of toxoflavin as indicator gave a positive reaction for a 4-nitro-2-aminophenol.

We have found that yeast lactic dehydrogenase + lactate will not take up oxygen in the presence of varying concentrations of dinitro-*o*-cresol in the Warburg apparatus. De Meio and Barron [1934] have just reported similar results with the α-hydroxy-oxidase of gonococci. This is readily explained by the fact that nitrophenols cannot be reduced by the lactate system in the absence of carriers, apart from any considerations of reversibility.

(4) *Succinic dehydrogenase.* The normal potential of the succinate-enzyme-fumarate system has been found to be near 0 v. at p_H 7 [Lehmann, 1929; Borsook and Schott, 1931]. It could hardly be expected that such a relatively positive system would be able to reduce nitrophenols readily even in presence of indicators. It was indeed found that a strongly active succinic dehydrogenase solution prepared according to Lehmann's directions, 0·5 ml. of which decolorised 0·3 ml. 1/2000 methylene blue in 15 mins. at 38°, failed to reduce dinitro-*o*-cresol either alone or in presence of the dyestuffs brilliant cresyl blue or thionine, although these dyes were completely decolorised.

As with the lactic dehydrogenase system, we tested the capability of dinitrocresol to provoke an oxygen uptake in the succinic dehydrogenase-succinate system. Washed chopped guinea-pig muscle, in the presence of cyanide to inhibit the normal oxygen utilisation, failed to take up oxygen in the presence of succinate when concentrations of dinitro-*o*-cresol varying between $2 \cdot 10^{-3}$ and $5 . 10^{-7} M$ were added (Fig. 4).

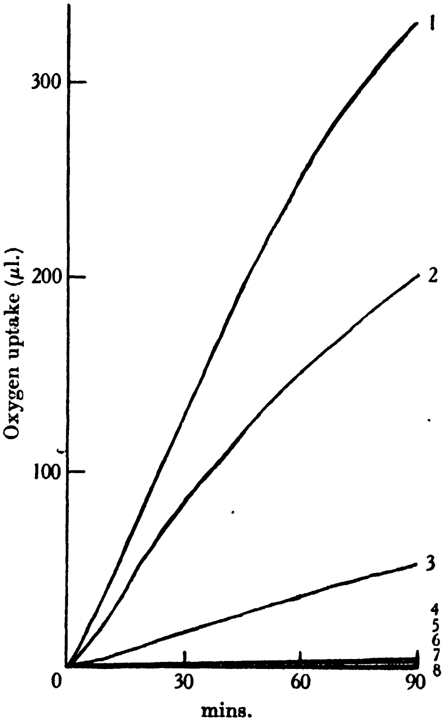


Fig. 4. Washed chopped guinea-pig muscle.

1. Succinate.
2. Succinate $+ 5 \times 10^{-3} M$ KCN $+ 2 \times 10^{-3} M$ methylene blue.
3. Succinate $+ 5 \times 10^{-3} M$ KCN $+ 2 \times 10^{-4} M$ methylene blue
4. Succinate $+ 5 \times 10^{-3} M$ KCN $+ 10^{-4} M$ dinitrocresol.
5. Succinate $+ 5 \times 10^{-3} M$ KCN $+ 10^{-5} M$ dinitrocresol.
6. Succinate $+ 5 \times 10^{-3} M$ KCN.
7. Succinate $+ 5 \times 10^{-3} M$ KCN $+ 2 \times 10^{-3} M$ dinitrocresol.
8. Succinate $+ 5 \times 10^{-3} M$ KCN $+ 5 \times 10^{-7} M$ dinitrocresol.

(5) *Reduction by muscle.* Unwashed chopped rat muscle contains systems which reduce benzyl viologen. Dinitro-*o*-cresol is likewise reduced, and the rate

of reduction is increased in the presence of benzyl viologen. Washing the muscle decreases its capability of reducing dinitrocresol, but the rate of reduction is still increased by the addition of benzyl viologen. We have not yet ascertained what systems are responsible for these reductions.

TECHNIQUE.

Experiments with Thunberg tubes. The vacuum tubes used in these experiments were mostly of the type shown in Fig. 5. They incorporate Keilin's hollow stopper, and in addition they are provided with glass collars which, together with the side arms, are filled with liquid paraffin. Fig. 5 also shows the arrangement used for alternately evacuating and refilling the tubes with nitrogen from a cylinder. The three-way tap T is first turned so that the Thunberg tube is connected with the water pump. When the liquid in the tube is boiling, the valve of the nitrogen cylinder is opened and the tap T is so turned that nitrogen passes into the Thunberg tube. The capillary constriction C prevents the rushing in of water from the safety device S. Evacuation and refilling with nitrogen are then repeated, and the tube is finally evacuated by leaving it in connection with the pump for 1 or 2 minutes.

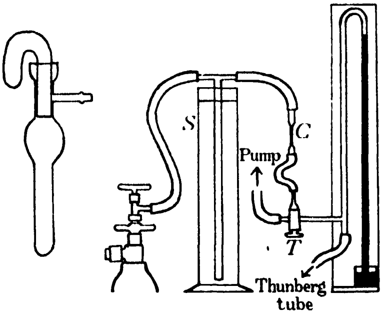


Fig. 5.

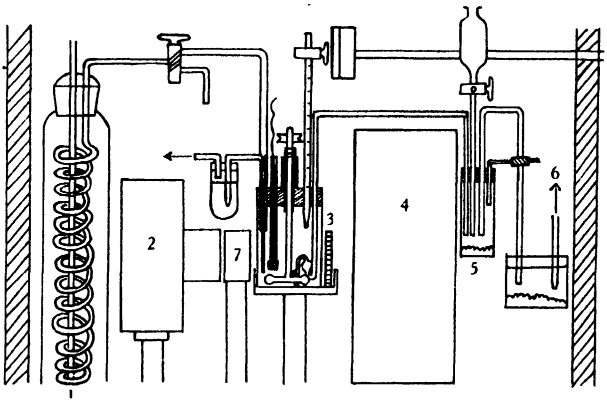


Fig. 6. 1. Pre-heating tower. 2. Photo-electric cell. 3. Light filters. 4. Light source. 5. Siphon system. 6. Calomel cell. 7. Diaphragm.

Potentiometric and photometric measurements. The apparatus illustrated in Fig. 6 allows simultaneous measurements of potential changes and of changes in light transmission to be made. The electrode vessel was fitted up in the manner described by Clark and Cohen [1923]. Two electrodes were used, one being of blank platinum foil and the other of the Westhaver type (platinum film on glass). Connection with the saturated calomel electrode was made by means of a syphon system [*cf*. Elema, 1931]. Before entering the titration vessel the gases passed through the pre-heating tower. Gas purification, temperature control and potential measurements were carried out as previously described [Stern, 1934, 1]. The electrode vessel was of square section with polished surfaces, the breadth being 4 cm. It was mounted on an optical bench between a 100-watt opal metal filament lamp and a photo-electric cell of the selenium type (Weston Photronic Cell). The light was filtered by means of a suitable combination of Schott coloured glass filters before it entered the titration vessel. The three filters used, each 1 mm. thick, were BG 17, 13, and 10. The photo-electric potentials, which varied between 1 and 14 mv., were measured with the same potentiometer and galvanometer as were used for the electrode potential measurements, the higher sensitivity range of the potentiometer being selected. The light source was switched on about 20 secs. before each reading. Stray light was eliminated by suitable housings for light source and photo-cells and by the provision of diaphragms.

DISCUSSION.

It is proposed first to discuss the question of the reversibility of the reduction of dinitrophenols. The reason for taking nitroaminophenol as the reductant in our potentiometric experiments was that the orange-red colour due to this compound begins to appear right from the beginning of reductive titrations. We have controlled this point by cautious titration of dinitrocresol with leucobenzyl viologen. The brownish colour appeared at once. Furthermore nitroaminophenol has been isolated as a product of the reduction of dinitrophenol by mild agents. Attainment of this stage, however, involves six hydrogen equivalents. No reversible system is known to be concerned with the exchange of more than two hydrogen equivalents in a single step. Since no typical reduction curves could be traced with dinitrocresol, no conclusions can be drawn from their slope as to the number of hydrogen equivalents involved.

Very probably all so-called irreversible systems possess a reversible phase in their oxidation or reduction as the case may be. The possibility of its detection is dependent upon the life time of the primary reaction product, which may usually be depicted as a radical liable to decomposition or secondary condensation. The refinement of methods in recent years has led to the demonstration of such reversible phases in systems hitherto regarded as irreversible. The rate of the disappearance of the radical determines the method to be chosen. Though the simple methods applied by us have failed, it may well be that by the application of such methods as the flow method [Ball *et al.*, 1933] or the method of discontinuous titration [Fieser, 1930, 1] a reversible stage may still be demonstrated in the reduction of dinitrophenols. From our data the possibility that dinitrophenol together with nitroaminophenol forms a sluggishly reversible system, comparable with the haemoglobin-methaemoglobin system, would appear to be ruled out.

For the time being Fieser [1930, 2] has recommended the method of potentiometric indicators for systems in which reversibility cannot yet be demonstrated. The critical oxidation potential introduced by this author is a unique function of the normal potential, by analogy with systems where both constants could be measured. Our experiments with potentiometric indicators suggest that the critical reduction potential of dinitrocresol is in the neighbourhood of −0·1 v. If it be assumed that there exists a reversible phase in the reduction of dinitrocresol involving two hydrogen equivalents, then following the deductions of Fieser one would arrive at a normal potential near −0·2 v., which would be in the region of the so-called anaerobic reduction potential of cells as determined by various methods (for literature see Stern [1934, 2]).

The experiments with the enzyme systems tried show that dinitrophenols can be reduced by enzyme systems which cover a wide range of reduction intensity provided a suitable carrier be present. It is not impossible that the cell contains such a carrier [see however Green, Stickland and Tarr, 1934]. With xanthine oxidase no extraneous carrier was necessary. We do not know whether this system contains already an appropriate carrier or whether it does not need one [*cf.* Green and Dixon, 1934]. The reduction of dinitrophenols in this respect parallels that of nitrate which is reduced by xanthine oxidase in the absence of a carrier, whereas its reduction by Stickland's preparation demands the presence of an added indicator [Green, Stickland and Tarr, 1934].

Our experiments show that dinitrophenols are not available to the dehydrogenases tried, with the exception of the xanthine oxidase system. An apparent inconsistency in our results concerning the coupled reduction of dinitrocresol demands comment. This compound is reduced in the presence of formic dehydrogenase-formate by an indicator, brilliant cresyl blue, which even in the 99 % reduced state does not possess a potential level low enough to cause an appreciable rate of reduction, as judged from our potentiometric indicator results. We would remark, however, that (1) the conditions in the potentiometric indicator and in the Thunberg tube experiments are not entirely comparable; (2) the potential of a fully reduced indicator is theoretically $-\infty$; (3) a leuco-dye formed by reduction on an enzyme surface is possibly in an "activated" state which may be very different from that of a dye reduced by non-enzymic agents.

We would emphasise the importance of arriving at an explanation of the action of nitrophenols on the living cell. In the stimulation which they produce, there is an overcoming of the processes which normally limit the respiration, and in some cases the glycolysis, of normal and tumour tissues. If the effect of the nitrophenols could be referred to their behaving as reversible redox systems, their action would be brought into line with that of reversible dyestuffs such as methylene blue, pyocyanin and thionine. If, on the other hand, the mechanism of their action were quite different, the possibility would arise that the reversible dyestuffs which stimulate the metabolism of tissue do so in virtue of some property not necessarily dependent on the reversibility of their reduction or the autoxidisability of their leuco-forms.

Whilst the work described in this paper was being completed, two communications appeared which give hypotheses for the mode of action of nitrophenols. De Meio and Barron [1934] state: "...the increase in respiration produced by dinitrophenol is not due to direct oxidation of the oxidisable substrates. It is suggested that dinitrophenol acts by combining with some of the substances acting as agents for the control of the speed of cellular oxidations, thus increasing the activity of the oxidising enzymes". On the other hand, according to Krahl and Clowes [1934], it is likely that dinitrocresol "stimulates cellular respiration by accelerating the oxidation by cytochrome of some substrate previously or simultaneously acted upon by the anaerobic dehydrases of the cell. It is too early to say whether DNC acts as a diffusible oxygen carrier between cytochrome and the substances normally oxidised or as an artificial substitute for a co-enzyme in the activation of substrates which do not normally play an important rôle in respiration".

We do not propose to comment upon these views, save to remark that De Meio and Barron's most weighty evidence for their views, namely that dinitrophenol is without effect when the respiration of cells and tissues has been inhibited by cyanide or carbon monoxide, does not exclude the possibility that the nitrophenols are converted into reversible systems in the presence of actively functioning oxidases or other cyanide-sensitive systems of the cell.

This paper does not attempt to explain the mechanism of nitrophenol action. It can, however, be said that, if the action of the nitrophenols depends on their reduction, a way in which such reduction may occur in the cell has been indicated.

SUMMARY.

1. The reduction of nitrophenols by enzymic and non-enzymic means has been studied, in the hope of obtaining an insight into the action of these compounds on the cell.

2. No evidence has been obtained that the reduction of dinitrophenols by agents such as hydrosulphite or palladium-hydrogen is reversible. The possibility of detecting a reversibility by more refined means than those employed is discussed.

3. The approximate potential level at which the reduction of dinitro-*o*-cresol becomes apparent has been determined by the method of potentiometric indicators.

4. Dinitrophenols are reduced by xanthine oxidase, formic dehydrogenase and lactic dehydrogenase in the presence of the appropriate substrates. With the last two enzymes, a small quantity of a reversible redox system must be added before reduction occurs. The product of reduction of 2:4-dinitrophenol by formic dehydrogenase-formate is 4-nitro-2-aminophenol.

5. No theory for the action of nitrophenols on cellular respiration is advanced; but the significance of the results is discussed.

It is a pleasure to acknowledge the interest that Prof. E. C. Dodds has taken in these experiments. We wish to thank him and the Council of the Middlesex Hospital Medical School for the hospitality afforded. To Dr Paul Fildes and to Dr G. P. Gladstone we are indebted for bacteriological assistance and advice.

REFERENCES.

Ball, Chen and Clark (1933). *J. Biol. Chem.* **102**, 691.
Barron (1930). *J. Exp. Med.* **52**, 447.
Bernheim (1928). *Biochem. J.* **22**, 1178.
Borsook and Schott (1931). *J. Biol. Chem.* **92**, 535.
Cannan, Cohen and Clark (1926). *U.S. Pub. Health Rep. Suppl.* **55**.
Chang and Gerard (1931). *Amer. J. Physiol.* **97**, 511.
Clark and Cohen (1923). *U.S. Pub. Health Rep.* **38**, 933.
Conant and Lutz (1923). *J. Amer. Chem. Soc.* **45**, 1047.
De Meio and Barron (1934). *Proc. Soc. Exp. Biol. Med.* **32**, 36.
—— Kissin and Barron (1934). *J. Biol. Chem.* **107**, 579.
Dickens (1934). *Nature*, **134**, 382.
Dixon and Kodama (1926). *Biochem. J.* **20**, 1104.
Dodds and Greville (1933). *Nature*, **132**, 966.
—— —— (1934). *Lancet*, i, 398.

Ehrenfest and Ronzoni (1933). *Proc. Soc. Exp. Biol. Med.* **31**, 318.

Elema (1931). *Rec. Trav. Chim. Pays-Bas.* **50**, 807.

Field, Martin and Field (1933). *Proc. Soc. Exp. Biol. Med.* **31**, 56.

—— —— —— (1934). *J. Cell. Comp. Physiol.* **4**, 405.

Fieser (1930, 1). *J. Amer. Chem. Soc.* **52**, 4915.

—— (1930, 2). *J. Amer. Chem. Soc.* **52**, 5204.

Filitti (1933). *Comp. Rend. Acad. Sci.* **197**, 1212.

Fleischmann and Kann (1933). *Biochem. Z.* **257**, 293.

Friedheim (1932). *Naturwiss.* **20**, 171.

—— (1934). *Biochem. J.* **28**, 173.

Gerard (1931). *Amer. J. Physiol.* **97**, 523.

—— (1932). *Physiol. Rev.* **11**, 469.

Green and Dixon (1934). *Biochem. J.* **28**, 237.

—— Stickland and Tarr (1934). *Biochem. J.* **28**, 1812.

Guerbet (1932). *Ann. Physiol. Physicochim. Biol.* **8**, 92.

—— and Mayer (1932). *Ann. Physiol. Physicochim. Biol.* **8**, 117.

Harrop and Barron (1928). *J. Exp. Med.* **48**, 207.

Krahl and Clowes (1934). *Proc. Soc. Exp. Biol. Med.* **32**, 226.

Lehmann (1929). *Skand. Arch. Physiol.* **58**, 173.

Michaelis and Hill (1933). *J. Gen. Physiol.* **16**, 859.

—— and Salomon (1931). *Biochem. Z.* **234**, 107.

Stern (1934, 1). *Biochem. J.* **28**, 949.

—— (1934, 2). *Tab. Biol. Period.* **4**, 1.

—— (1935). *Biochem. J.* **29**, 500.

Stickland (1929). *Biochem. J.* **23**, 1187.

LVII. OXIDATION-REDUCTION POTENTIALS OF TOXOFLAVIN.

By KURT GUENTER STERN.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

(*Received December 21st, 1934.*)

Toxoflavin is the name given by van Veen and Mertens [1934] to the prosthetic group of the yellow pigment which is formed in cultures of *Bacterium bongkrek* under aerobic conditions. The native pigment complex together with a colourless compound is thought by these authors to be responsible for the occurrence of a number of cases of foodstuff poisoning among the natives of Java. Whereas the native pigment is highly toxic when administered perorally or intraperitoneally to monkeys, pigeons and rats, toxoflavin in crystallised state has only retained its toxicity for rats when injected intraperitoneally, 5–25γ representing the lethal dose. The decrease in toxicity is ascribed by the authors to the detachment of the prosthetic group from some colloidal carrier substance in the course of purification.

The empirical formula assigned to toxoflavin is $C_6H_6O_2N_4$. The constitutional formula is unknown. Of the chemical properties reported in van Veen and Mertens's publication only the following may be mentioned here in view of their bearing on the present work. The compound (M.P. 171°) is readily soluble in water and alcohol, less soluble in ethyl acetate and chloroform, hardly soluble or insoluble in ether, benzene, and light petroleum. In water the yellow colour is only stable between p_H 3 and 7. Slow decoloration takes place at p_H 1 and 9. Addition of mineral acids or strong alkali causes immediate decoloration. Toxoflavin is stable towards oxidising agents (nitric acid, hydrogen peroxide, bromine water). In weakly acid or alkaline solution the yellow colour is discharged instantaneously by sulphurous acid and it returns on shaking with air or addition of hydrogen peroxide or bromine water. On catalytic hydrogenation according to the selected conditions either approximately one or three hydrogen molecules are taken up by one molecule. One methylimide group was found. The murexide test is only positive if potassium chlorate and hydrochloric acid are used instead of nitric acid. As oxidation products alloxan (or methylalloxan), methylamine and ammonia were found. In concentrated aqueous solution toxoflavin forms a brick-red, sparingly soluble addition compound with bisulphite. The solutions of toxoflavin, especially in amyl alcohol, are stated to exhibit a green fluorescence.

The idea of carrying out the experiments which are subsequently described arose from the conclusion drawn by van Veen and Mertens from their findings that toxoflavin was to be regarded as a member of the series of flavin or lyochrome pigments recently studied by Warburg, Ellinger, Kuhn, Karrer, von Euler and by the present author.

This investigation was made possible by the generous gift of about 20 mg. of pure recrystallised toxoflavin by Dr A. G. van Veen to whom sincere thanks are due. He consented to the potentiometric and spectrographic examination of the pigment[1], on lines along which detailed information in respect to flavin pigments was available from this laboratory [Holiday and Stern, 1934; Stern, 1934, 2].

EXPERIMENTAL.

Material. The sample of toxoflavin which was used for the following experiments was sent from Batavia-Centrum in a sealed ampoule. The colour of the crystals, which is much like that of picric acid, is stated to change somewhat to a brownish tint in a tropical climate when exposed to the air, but the crystals did not show any detectable change during their 4 months' storage in a stoppered tube in this laboratory.

The crystals were found to be free from moisture (3·04 mg. when subjected to a slow current of air, previously dried over calcium chloride, for 3 hours at 75° in a platinum boat in Pregl's micro-desiccator (suction applied by high pressure water-pump) did not show any loss of weight detectable with a Sartorius microbalance).

The properties were identical with those described by van Veen and Mertens with the exception of the fluorescence which was found to be hardly perceptible even in a strong beam of Wood's light when the substance was dissolved in water, chloroform or amyl alcohol. The weakness of the fluorescence became especially obvious when as controls similar solutions of photo-hepatoflavin or of 9-methylalloxazin were used.

The aqueous solution of toxoflavin reacts weakly acid. A 10^{-4} M solution showed a p_H of 6·5 when measured with the glass electrode.

Toxoflavin does not couple with diazobenzenesulphonic acid. No free NH_2-group is detectable with 1: 2-naphthoquinone-4-sulphonic acid.

Methods. The arrangement and method of potentiometric measurements were the same as described in a study dealing with photo-flavin [Stern, 1934, 2]. The potassium ferricyanide preparation used for oxidative titrations of leuco-toxoflavin was an analytically pure sample. It was free from ferrocyanide, and on titration with permanganate after reduction with sodium peroxide 100 % of the calculated iron content was found. For the purpose of reductive titrations the technique described by Clark and Perkins [1932] was employed. The stock solution of chromous acetate was stored under hydrogen and delivered from a Bang microburette. To the tip of this burette a small vessel containing the necessary amount of buffer solution was attached. After thorough de-aeration with hydrogen or nitrogen the calculated amount of the stock solution was run into the buffer mixture. The receiving vessel was detached from the Bang burette and the diluted titrant was quickly covered with liquid paraffin. It was then drawn up into the actual microburette serving for the electrometric titrations protected by a paraffin seal. The strength of the chromous acetate stock solution was determined before and after the preparation of the dilution by means of a standardised ferric alum solution with ammonium thiocyanate as internal indicator.

The spectrographic measurements were made with the aid of a medium-size Hilger quartz spectrograph with attached Spekker quartz photometer. The condensed spark discharge between tungsten steel electrodes served as light source. The photographs were evaluated by the match point method.

For the manometric experiments the usual Barcroft-Warburg technique was employed.

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[1] A short account of this work was presented jointly with Holiday at the Meeting of the Biochemical Society on November 16th, 1934 [Stern and Holiday, 1934, 2].

RESULTS.

Oxidation-reduction potentials of toxoflavin.

Nineteen titration experiments were performed covering the p_H range from 2·68 to 8·49. In accordance with the qualitative findings of van Veen and Mertens the stability of the pigment was sufficient for reproducible measurements only between p_H 4·6 and 8·3. At p_H 2·68 the oxidised (yellow) form of the system became unstable. At p_H 4·12 and 8·49 the reduced form was not sufficiently stable to allow the tracing of a definite titration curve. Between p_H 4·6 and 8·3 toxoflavin represents the oxidant of a stable, reversible and electro-active redox system. Titrations of the leuco-form with ferricyanide and of the oxidant with chromous acetate yield substantially consistent results. In the poising range of the system equilibrium is almost instantaneously reached, the potentials are stable in time, and individual electrodes differ seldom by more than 1 mv. Generally they agree in the range from 20 to 80 % oxidation within 0·1 mv.

Potential range. The normal potentials of toxoflavin as obtained by graphical interpolation from the individual titration curves at varying hydrogen-ion concentrations are given in Table I in column "E_0'". The potentials were measured against the saturated calomel half cell and the values with reference to the normal hydrogen electrode were then calculated therefrom. The curve combining these E_0' values is plotted in the right-hand part of the composite diagram in Fig. 1. It will be seen from this curve that the toxoflavin system is much more positive than the flavin systems studied by the writer. For instance, whereas E_0' for toxoflavin at p_H 7 is $-0·049$ v., the corresponding value for photo-hepatoflavin is $-0·227$ v. [Stern, 1934, 2] and for hepato-, malto- and uro-flavin the mean value is $-0·215$ v. [Stern, 1934, 1]. On the other hand the toxoflavin potential at p_H 7 is very near to the pyocyanin potential ($-0·034$ v. [Friedheim and Michaelis, 1931; Elema, 1931]).

Table I. *Results of titrations of 10^{-4}*M *leuco-toxoflavin with $2·10^{-3}$*M *potassium ferricyanide (30°).*

(Potential values referring to the normal H_2 electrode.)

p_H	Buffer	$E_{\frac{1}{4}}$	$E_0'=E_{\frac{1}{2}}$	$E_{\frac{3}{4}}$	E_1	0·03 log K $E_M-E_1=E_2-E_M$	(after Michaelis)		K (after Elema)
							log K	K	
4·65	Acetate	+0·036	+0·058	+0·081	0·0225	Mean ∼0	±0	±1	—
5·22	Veronal-acetate-HCl	+0·0015	+0·0245	+0·0495	0·024				
5·41	,,	−0·007	+0·014	+0·037	0·022				
5·69	,,	−0·019	+0·001	+0·023	0·021				
6·07	,,	−0·0365	−0·0185	+0·0015	0·019				
6·36	,,	−0·043	−0·024	−0·005	0·019				
6·82	,,	−0·064	−0·040	−0·015	0·0245	+0·0085	+0·283	1·91	1·39
7·37	Phosphate	−0·090	−0·059	−0·029	0·0305	+0·021	+0·7	5·01	4·56
8·09	Veronal-acetate-HCl	−0·1085	−0·0735	−0·0395	0·0345	+0·028	+0·93	8·51	10·1
8·30	,,	−0·110	−0·077	−0·042	0·034	+0·0275	+0·917	8·26	10·82

The shape of the E_0'/p_H curve for toxoflavin reveals the existence of two dissociation constants in the range of stability of both components of the system: p_{K_1} about 5·8 and p_{K_2} about 7·2.

The slope of the curve from p_H 4·6 to 5·7 is $\frac{-\Delta E}{\Delta p_H}=60$ mv., from p_H 6·1 to 7 = 35 mv., and from p_H 7·4 to 8·3 = 19 mv. The changing of the slope of the curve, due to p_{K_1}, by approximately 30 mv. suggests that the nature of the system is a "two electron-system" ($n=2$). p_{K_2} however changes the slope in an atypical manner. This may possibly be explained by the assumption that the part of the curve extending from p_H 7·4 to 8·3 is eventually tending to a zero mv. slope. This hypothesis however cannot be tested experimentally in view of the instability of the system beyond p_H 8·3.

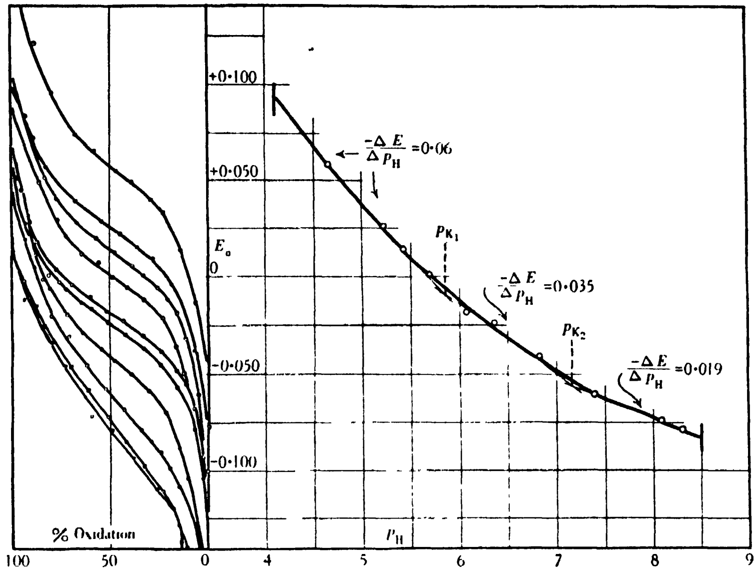

Fig. 1. Toxoflavin: Relation of E_0' to p_H.

The experiments which served for the compilation of Table I as well as for the diagram (Fig. 1) were all done by titrating leuco-toxoflavin with potassium ferricyanide.

Electron number. As will be seen by inspection of the individual titration curves plotted in the left-hand side of Fig. 1 as well as from the column "E_i" of Table I, the slope of these curves is throughout an atypical one. This is best demonstrated by the value of the so-called index potential (E_i), *i.e.* the potential difference between 50 and 25 % (or 75 % respectively) oxidation. Whereas E_i for a system losing two electrons in a single step on oxidation, which is the most common type of organic redox system, would amount to 14 mv., E_i for a one-electron system like cytochrome would be 28 mv.

With the present system E_i averages 21 mv. between p_H 4·6 and 6·4, and then increases with p_H, to become 34 mv. at p_H 8·3. A variation of E_i with p_H is also shown by the two step reduction systems recently studied by Michaelis [1933] and by Elema [1931; 1933], *e.g.* pyocyanin and chlororaphin. The flavins have been shown to belong to the same class of systems [Stern, 1934, 2]. A fundamental difference between the above-mentioned systems and toxoflavin however is the fact that the steepening of the titration curves and simultaneous visible semiquinone formation in the case of pyocyanin etc. is observed in the acid range, whereas with toxoflavin the change occurs in the alkaline range without any visible semiquinone formation[1].

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[1] The only similar case of which the writer is aware is that of the pigment of *Halla parthenopea*—hallachrome—which was recently studied by Friedheim [1933]. Here E_i is about 20 mv. from p_H 0·19 to 9·8. Further towards the alkaline range E_i increases and becomes 34 mv. at p_H 11·47.

It follows therefore that the number of electrons involved in the process of oxidation-reduction of toxoflavin cannot be derived from the slope of individual titration curves. If the view be accepted that we are dealing here with a two-step reduction system, though no formation of an intermediary form may be detected by a colour change on reduction and though no complete separation of the two steps involved could be realised owing to the overlapping of the steps within the p_H range of stability, the complete process of reduction would consist in the uptake of two hydrogen equivalents or electrons. This conclusion is supported by the above-mentioned change in the slope of the E_0'/p_H curve of approximately 30 mv. by the first dissociation constant of the reduced form, and also by the observation of van Veen and Mertens that either one or three molecules of hydrogen are taken up by toxoflavin according to the conditions chosen. The uptake of one H_2 molecule would correspond to the reversible process amenable to electrometric study.

Treatment of toxoflavin as a two-step reduction system.

Assuming that toxoflavin is reduced in two steps increasingly overlapping towards the acid range and each involving the uptake of one electron, the process may tentatively be subjected to theoretical treatment along the lines developed by Michaelis and independently by Elema. These authors have shown how the distance of the normal potentials of the two single steps of such a system from the midpoint of the whole titration curve may easily be determined even in the range of considerable overlapping. Furthermore they have made available short cuts for obtaining the value of the characteristic "effective formation constant of the intermediary form (semiquinone)" at any given p_H value. The magnitude of this constant, K, is an adequate representation of the probability of free radical (semiquinone) formation and also of its stability. Michaelis [1933] for this purpose has compiled a table correlating the experimentally observed E_1 values with the distance of the separate normal potentials ($E_M - E_1 = E_2 - E_M$) from the midpoint of the titration curves and with $\log K$. Elema [1933] on the other hand has shown that a simple relation holds between K and the tangent to the titration curve at the midpoint. The height of ordinate defined by the midpoint of the curve and by the point of intersection of the tangent with the ordinate is called S. Then $K = (76{\cdot}63 . S - 2)^2$. In Table I both methods have been applied to the data obtained for toxoflavin. Since the intervals in the table of Michaelis are somewhat too large for simple interpolation ($E_M - E_1 = 0{\cdot}03 \log K$ being no linear function of E_1) a diagram was first constructed from the table of Michaelis and the values given in Table I were obtained by graphical interpolation. As will be seen from Table I, there is a general agreement between the K values derived by the methods of Michaelis and of Elema. Theoretically the agreement between the results of the two methods should be closer, which implies the conclusion that the differences actually found are to be ascribed to experimental errors.

Molecular weight of toxoflavin.

On the ground of molecular weight determinations by the cryoscopic method (solvent, water) and by Rast's method (solvent, camphor) van Veen and Mertens assign to toxoflavin the formula $C_6H_6O_2N_4$. The Rast determination is however invalidated by decomposition of the pigment in melting camphor. It was therefore thought to be desirable to obtain information about the molecular weight of toxoflavin by an independent method. In the case of pyocyanin Friedheim and Michaelis [1931] were able by the mere slope of the titration curves to establish the true molecular weight as half the value given by Wrede and Strack. In the case of toxoflavin where the slope of the titration curves is atypical throughout the whole p_H range the stoichiometric relations had to be taken into consideration. The basic assumption to be made was that two hydrogen equivalents are concerned in the reversible process of oxidation-reduction. No decision could be reached by oxidative titration experiments with potassium ferricyanide. In these experiments 10 ml. toxoflavin solution containing 0·166 to 0·167 mg. were reduced with palladium-hydrogen and then titrated with a $2{\cdot}10^{-3}\,M$ ferricyanide solution. For $n=2$ there should have been required 0·5 ml. ferricyanide if the molecular weight was 332, and 1·0 ml. for a molecular weight of 166. Actually the end-points observed on electrometric titration fell invariably near 0·7 ml. ferricyanide. The same irregularity has previously been observed in the case of photo-hepatoflavin [Stern, 1934, 2]. Better results were obtained by means of reductive titrations with chromous acetate. 10 ml. toxoflavin solution containing 0·166 mg. were titrated at p_H 5·67 with $5{\cdot}35.10^{-3}\,M$ chromous acetate solution. If the molecular weight of toxoflavin were 332, 0·187 ml. of the titrant, and if it were 166, 0·374 ml. should be required. As determined from the titration graph 0·32 M chromous acetate solution was necessary. In a similar experiment at p_H 4·6 the quantity of titrant used was 0·325 ml. of a $5{\cdot}16.10^{-3}\,M$ chromous acetate solution (calculated for mol. wt. 166:0·387 ml., for mol. wt. 332:0·193 ml.).

These experiments are therefore corroborative evidence in favour of the formula of toxoflavin proposed by the discoverers.

Results of spectrographic study.

The measurements of light absorption have been carried out by Dr Holiday at the Laboratories of the Medical Unit of the London Hospital Medical School [*cf.* Stern and Holiday, 1934, 2]. Their result was that a general resemblance exists between the patterns of the flavin and of the toxoflavin spectra. In aqueous solution (p_H 6·5) a steep main absorption band at 260$m\mu$ is accompanied by an inflection at 310$m\mu$ and by a lower and rather broad band centred at 405$m\mu$. The molecular extinction coefficients calculated for a mol. wt. of 166 amount

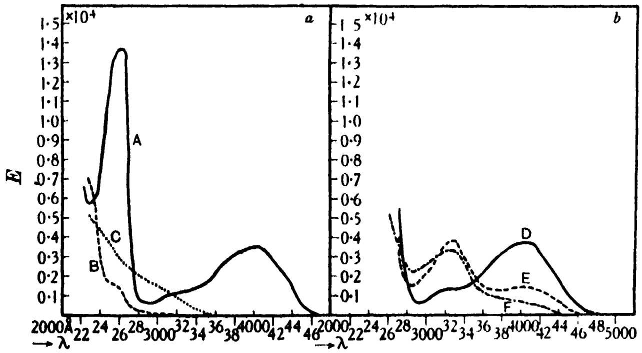

Fig. 2. Spectral behaviour of toxoflavin. Ordinates: mol. extinct. coeff. Abscissae: wave-lengths in Å.

A. ——— p_H 6·5.
B. - - - - p_H 3·0 after standing.
C. ······ p_H 11·0 after standing.
D. ——— p_H 6·5.
E. - - - - p_H 9·0 immediately after dissolving.
F. -·-·- p_H 11·0 immediately after dissolving.

to about one-half of the corresponding values for flavins. After standing for a short time at p_H 3 or p_H 11 the fading of the yellow colour is accompanied by an almost complete disappearance of specific light absorption. These results are illustrated in Fig. 2. At p_H 9 the height of the long wave band decreases and a distinct band is formed instead at $320 m\mu$. This band also slowly disappears with time.

The disappearance of specific absorption in acid and in alkaline solution seems to indicate an opening of a ring structure in the molecule.

Effect of toxoflavin on cell respiration.

Judged from the value of its normal potential toxoflavin should increase the oxygen uptake of non-nucleated red blood corpuscles to a similar extent to *e.g.* methylene blue or pyocyanin, assuming of course that the effect is not obscured by secondary factors such as toxicity or diffusibility.

Manometric experiments have been carried out by Mr Greville with human and rabbit erythrocytes. The result was that toxoflavin stimulates their respiration to about the same extent as thionine ($E_0' = +0{\cdot}062$ at p_H 7). The course of an experiment with rabbit blood corpuscles is illustrated in Fig. 3. In view of current discussion on the mechanism of stimulation of respiration in erythrocytes by oxidation-reduction systems it may not be without interest to mention that toxoflavin is capable of transforming oxyhaemoglobin with fair velocity into methaemoglobin.

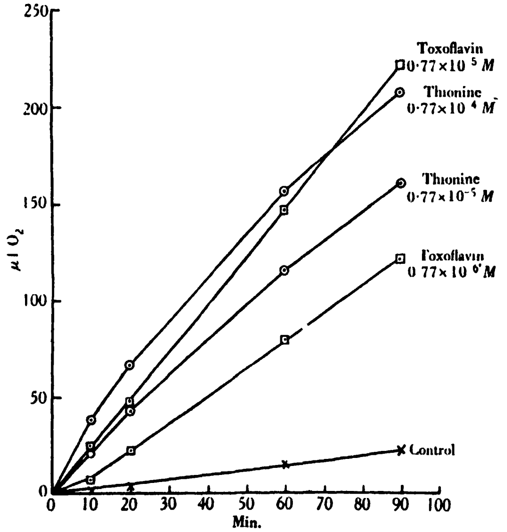

Fig. 3. Effect of toxoflavin and of thionine on respiration of rabbit erythrocytes in 0·2 % glucose.

The effect of toxoflavin on the respiration of Jensen rat sarcoma and on normal tissues (*e.g.* brain) is under investigation. Evidence so far available indicates that the respiration of Jensen rat sarcoma in glucose is also increased by toxoflavin (140 % increase by 2.10^{-5} mol. toxoflavin). The effect however decreases rapidly, probably because of a toxic action of the pigment which is shown better by brain slices, where a concentration of 10^{-5} mol. already strongly inhibits the respiration.

Discussion.

It is proposed to consider first the bearing of the results of this investigation on the question of a structural relationship between toxoflavin and the group of flavin (lyochrome) pigments. Van Veen and Mertens were led to assume such a relationship on the ground of similarities of a more qualitative nature, *e.g.* colour, fluorescence, stability towards oxidising agents and reversible reducibility. Though at the time of the completion of their paper (December, 1933) the flavin chemistry was still in its early stages, the authors were well aware of certain fundamental differences between toxoflavin and the other flavin pigments, *e.g.* in respect to behaviour towards radiation, formation of sparingly soluble silver salts and nitrogen content. In spite of the fact that the exact constitution of toxoflavin itself is still unknown, the rapid progress of flavin chemistry since the publication of Van Veen and Mertens's paper together with the data made available by the present experiments should furnish a definite answer to that question. If at the outset there existed a link of comparison between toxoflavin and the class of flavin pigments, such a link could only be seen in the photoflavins (or lumi-flavins), *i.e.* in the photo-decomposition products of the native flavins, but not in the native pigments themselves. Toxoflavin shares with a photo-flavin the solubility both in water and in chloroform, the stability towards oxidants, the reversibility of reduction and the presence of a methylimide group. There exists also a similarity in the general pattern of the absorption spectrum. On a closer examination of the data obtained on photo-flavins [Stern, 1934, 2] and on toxoflavin (this paper) with the same methods, these similarities turn out to be of a rather superficial nature. The major points are brought out in Table II.

Table II.

	Toxoflavin	Photoflavins
Range of stability	p_H 4 to 8	Conc. H_2SO_4—cold N NaOH
Normal potential at p_H 7	$E_0' = -0{\cdot}049$ v.	$E_0' = -0{\cdot}22$ v.
Slope of titration curves	Atypical throughout, increasing steepness towards alkaline range	Typical for $n=2$ in physiological range, increasing steepness toward acid range
Semiquinone formation	Not detectable; if any, then in alkaline range	Stable, red semiquinone in acid solution
Dissociation constants:		
p_K of reduced form	5·8 and 7·2	7·7
p_K of oxidised form	—	0·4 and 10
Maxima of light absorption	260; 310; 405 $m\mu$ (p_H 6·5)	267; 364; 440 $m\mu$ (p_H 7) (photo-hepatoflavin)

The data given in the column "Photoflavins" apply, with the noted exception of absorption bands, to the whole group of photoflavins, including those formed on irradiation of native flavins (discovered by Warburg and Christian [1932]) and the synthetic members of this group [Stern and Holiday, 1934, 1]. For further material for comparison in respect to the spectral behaviour of toxoflavin and the photoflavins, the reader is referred to the paper by Holiday and Stern [1934].

There are also several points of biological interest which deserve discussion. Whereas it is shown in this paper that toxoflavin in accordance with its potential may act as a stimulant after respiration of mammalian red blood cells and may convert oxyhaemoglobin into methaemoglobin, flavins do not possess such properties [Wagner-Jauregg *et al.*, 1933; 1934].

According to the description given by van Veen, toxoflavin seems to be present also outside the bacterium in the culture medium, though linked up to a high molecular complex. On the other hand bacterial flavin pigments have been found exclusively inside the cell in a non-diffusible form. Their concentration seems to be highest in strictly anaerobic organisms [Warburg and Christian, 1933], whereas toxoflavin is said not to be formed under anaerobic conditions. Finally, the flavin pigments so far investigated have all been found to be non-toxic.

It appears therefore that whatever similarity may have brought about the tentative classification of toxoflavin among the flavin series it is at present so much outweighed by fundamental differences between toxoflavin and all the known flavins, that this assumption were better abandoned. To our mind toxoflavin appears to be a new and interesting bacterial product of yellow colour without structural or functional relationship to the flavin pigments. The neighbourhood of its normal potential to that of pyocyanin suggests a similar function, probably that of an accessory respiratory catalyst [*cf.* Friedheim, 1931]. It is not unlikely that *Bacterium bongkrek* apart from toxoflavin will be found to contain a real flavin pigment concerned with a different phase of metabolism.

SUMMARY.

Toxoflavin, the prosthetic group of a yellow pigment formed by *Bacterium bongkrek* (van Veen and Mertens) represents the oxidant of an oxidation-reduction system which is fully reversible and electro-active between p_H 4 and p_H 8. The normal potential, as referred to the normal hydrogen electrode, at p_H 7·0 is $-0·049$ v. The slope of the individual titration curves is atypical throughout the p_H range investigated and shows an increasing steepness towards the alkaline range. Though no colour change indicating semiquinone formation was observed, the experimental data have tentatively been subjected to theoretical interpretation in the light of the theory of Michaelis and Elema of two-step reduction. The relation of toxoflavin to the group of flavin pigments is discussed.

The author wishes to thank Prof. E. C. Dodds for his interest and encouragement. Furthermore he wishes to thank him and the Council of the Middlesex Medical School for the hospitality afforded.

REFERENCES.

Clark and Perkins (1932). *J. Amer. Chem. Soc.* **54**, 1228.
Elema (1931). *Rec. Trav. Chim. Pays Bas*, **50**, 807.
—— (1933). *Rec. Trav. Chim. Pays Bas*, **52**, 569.
Friedheim (1931). *J. Exp. Med.* **54**, 207.
—— (1933). *Biochem. Z.* **259**, 257.
—— and Michaelis (1931). *J. Biol. Chem.* **91**, 355.
Holiday and Stern (1934). *Ber. deutsch. chem. Ges.* **67**, 1352.
Michaelis (1933). Oxydations-Reduktions-Potentiale, 2nd ed., p. 107.
Stern (1934, 1). *Nature*, **133**, 178.
—— (1934, 2). *Biochem. J.* **28**, 949.
—— and Holiday (1934, 1). *Ber. deutsch. chem. Ges.* **67**, 1104, 1442.
—— —— (1934, 2). *Chem. Ind.* **53**, 1025.
van Veen and Mertens (1934). *Rec. Trav. Chim. Pays Bas*, **53**, 257, 398.
Wagner-Jauregg and Ruska (1933). *Ber. deutsch. chem. Ges.* **66**, 1298.
—— and Wollschitt (1934). *Naturwiss.* **22**, 107.
Warburg and Christian (1932). *Biochem. Z.* **254**, 438.
—— —— (1933). *Biochem. Z.* **266**, 377.

LVIII. THE INACTIVATION OF CATALASES FROM CERTAIN MARINE PLANTS BY OXYGEN.

By GRAHAM WALLACE MARKS.

Scripps Institution of Oceanography, University of California,
La Jolla, California.

(*Received November 2nd, 1934.*)

It has been shown [Marks and Fox, 1933; Marks, 1934, 1] that catalases from certain marine animals, on standing in air, slowly lose their ability to decompose hydrogen peroxide. This inactivation was found to be caused primarily by oxygen and the reaction to be pseudo-unimolecular under the given experimental conditions. The question arose as to whether or not catalases from marine plants would likewise be inactivated.

Methods.

The experimental procedure has already been described [Marks, 1934, 2]. Unless otherwise stated, the temperature at which extracts were stored and measurements were made was 20·0 ± 0·2°. The reaction time interval was 60 minutes.

Inactivation by oxygen. Solutions were prepared by extraction with a phosphate buffer of $p_{\rm H}$ 7·0, unless otherwise specified, of a number of species of marine plants. Portions were stored in air and under nitrogen or oxygen or both. Daily measurements were made on the quantity of hydrogen peroxide decomposed by 5 ml. samples after the addition of 2 ml. of the former. Controls were run simultaneously, using portions of extract that had been heated to boiling. The hydrogen peroxide solutions were prepared in the corresponding buffer or other corresponding medium and were 0·84–0·98 M.

It was found possible to apply the equation for a first order reaction to the inactivation reactions, as was previously done for other catalases [Marks, 1934, 1]. Thus, in the expression, $k=\frac{2\cdot303}{t}\log\frac{a}{a-x}$, let a = the initial number of mg. of hydrogen peroxide decomposed by 5 ml. of extract, and $(a-x)$ = the number of mg. of hydrogen peroxide decomposed by 5 ml. of extract at any time, t, in days after the start. It has been shown [Marks, 1934, 2] that the quantities of hydrogen peroxide decomposed by the catalases from *Corallina officinalis* and *Pikea californica* are directly proportional to the enzyme concentration at $p_{\rm H}$ 7·0. This has been assumed to be true for the catalases from other species of marine plants. The true concentration of enzyme would be given by multiplying a and $(a-x)$ by some unknown factor c. However, this cancels out in the logarithmic expression, and thus the procedure is justified.

Data are given in Table I and show that the rates of inactivation were always greater for the portions of extract kept under air than under nitrogen. However, since oxygen enters directly or indirectly into the inactivations, the reactions are pseudo-unimolecular [Taylor, 1930]. Thus, the true equation is $k=\frac{1}{tb}\ln\frac{a}{a-x}$, in which b is the concentration of oxygen or oxygen and unknown substances. This factor remains constant for any single experiment.

Table I. *The inactivation of catalases from marine plants.*

Species	Conc. of extract %	Medium	Atmosphere	No. of measurements*	*k* Range†	*k* Mean
Corallina officinalis	27	Water p_H 6·2	Air	11	0·076–0·093	0·085
			Nitrogen	8	0·026–0·034	0·033
Corallina officinalis	20	Phosphate buffer p_H 7·0	Oxygen	7	0·254–0·288	0·273
			Air	7	0·144–0·162	0·149
			Nitrogen	6	0·099–0·117	0·110
Corallina officinalis	40	Sea water p_H 8·1	Air	6	0·145–0·167	0·157
Pikea californica	20	Phosphate buffer p_H 7·0	Air	13	0·041–0·063	0·044
			Nitrogen		Rate slower‡	
Amphiroa aspergillum forma nana	40	Phosphate buffer p_H 7·0	Air	9	0·113–0·142	0·127
			Nitrogen	10	0·062–0·087	0·081
Corallina gracilis forma densa	20	Phosphate buffer p_H 7·0	Oxygen	7	0·154–0·186	0·168
			Air	9	0·131–0·147	0·136
			Nitrogen	9	0·046–0·052	0·049
Plocamium coccineum	35	Phosphate buffer p_H 7·0	Air	9	0·142–0·160	0·149
			Nitrogen	9	0·083–0·096	0·089
Polysiphonia verticillata	20	Phosphate buffer p_H 7·0	Air	5	0·262–0·273	0·267
			Nitrogen		Rate slower‡	

* Taken at 24 hour intervals.

† Although the range of *k* seems wide in some cases, actually most of the values clustered about the mean, and only one or two observations departed greatly therefrom.

‡ Rate did not conform satisfactorily to the unimolecular equation. Compare Marks and Fox [1933].

It was found that the catalases in 10–20 % extracts prepared from *Pelvetia fastigiata*, *Colpomenia sinnora*, *Dilophus flabellatus*, *Scytosiphon lomentarius* and *Sargassium agardianum* in a phosphate buffer at p_H 7·0 almost completely lost their activity on standing in air for 24 hours at 20°.

Inactivation at different p_H *values.* That the inactivation occurs irrespective of hydrogen ion concentration is shown in Fig. 1 for catalase from *Corallina officinalis*. It must not be inferred that because higher magnitudes of *k* are obtained at lower p_H values the reactions proceeded more rapidly. Reference to curves 1, 2 and 6 of Fig. 2 in the paper of Marks [1934, 2] shows that such results can be ascribed to the greater slope of portions of the proportionality curves. These portions do not pass through the origin. It is not now possible to say just what effect hydrogen ion and salt concentrations have on the reaction rates.

Previously, sharp changes in the slopes of the curves were noted when the data for the inactivation of catalases from certain marine animals were plotted logarithmically [Marks, 1934, 1]. Similar discontinuities (Fig. 1) have been found for the inactivation of catalase from *Corallina officinalis* at p_H 8·0 and 9·0. Again these changes are not to be ascribed to a difference in reaction rate, but to discontinuities in the proportionality curves. Thus, referring again to Fig. 2 [Marks, 1934, 2] for p_H 10·0, the change occurs at about 22 mg. on the ordinate, and in Fig. 1 (p_H 10·0) of the same paper, an apparent change in reaction rate is found at about 20 mg. ($\log_{10} = 1·30$), *i.e.*, both changes occur at approximately the same catalase concentration.

Inactivation of catalases at different temperatures. It is to be expected that the rate of inactivation will increase with rise in temperature. Extracts were prepared in phosphate buffer p_H 7·0, divided into several portions and stored at given temperatures which varied from their mean values by about ±0·2°.

Daily measurements were made at 20·0 ± 0·2°, the sample first being kept for 20–30 minutes in a room at this temperature before the introduction of hydrogen peroxide.

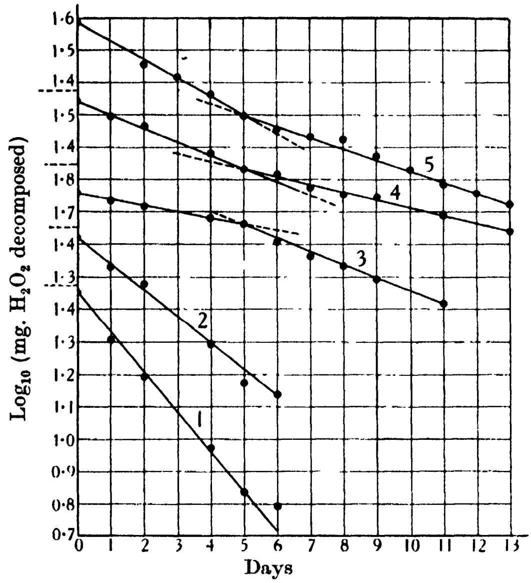

Fig. 1. Inactivation of catalase from *Corallina officinalis* at different p_H values. Respective concentrations of extracts and p_H of buffers used are in the order of the numbers of the curves: 40 %, 40 %, others 20 %; p_H 4·5, 6·0, 8·0, 9·0 and 10·0. All buffers phosphate except 9·0 and 10·0 which were H_3BO_3-KCl-NaOH mixtures.

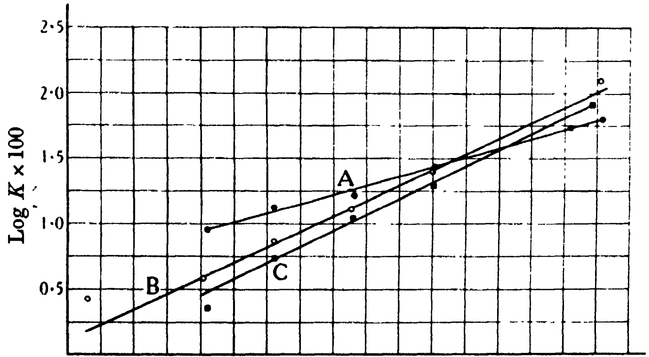

Fig. 2. Inactivation of catalases from marine plants. Curves showing the application of Arrhenius's equation for change in reaction rate with change in absolute temperature. Curves *A* and *B*, *Corallina officinalis* (20 % extracts); curve *C*, *Pikea californica* (40 % extract).

Initial values of *a* were determined by the extrapolation method [Marks and Fox, 1933]. These extrapolated values were not precisely the same for the different temperatures for a given extract, but only approximately so.

Application of Arrhenius's equation. Arrhenius's equation was previously found applicable to the change in inactivation rate with change in temperature for mussel catalase [Marks and Fox, 1933]. Since again it is assumed that unknown factors, including oxygen, remained constant under the given conditions, the integrated form of Arrhenius's equation [Taylor, 1930] may be written as follows:

$$2{\cdot}303 \log \frac{k_1}{k_2} = \frac{Q}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right),$$

where $k_1 = k'b$, and $k_2 = k''b$.

In Fig. 2 are plotted data obtained for catalases from *Corallina officinalis* and *Pikea californica*. Since the curves are straight lines, the Arrhenius equation is applicable. It is to be noticed that the slopes of curves *A* and *B* for two different extracts of *C. officinalis* having the same percentage concentration are quite different. Thus the values of *Q* are not the same in the two cases. This effect can be ascribed to an unknown factor or factors which remain constant for a given extract. It is hardly to be ascribed to oxygen alone as there is no reason for believing that the concentrations of this gas were significantly different in the two preparations at the different temperatures.

SUMMARY.

1. Catalases from certain species of marine plants are inactivated, directly or indirectly by oxygen and the reactions have been found to be pseudo-unimolecular under the given experimental conditions.

2. Catalase from *Corallina officinalis* slowly loses its activity over the p_H range 4·5–10·0 at 20·0 ± 0·2°.

3. The change in the rate of inactivation of catalases from *Corallina officinalis* and *Pikea californica* with change in temperature is expressed by the Arrhenius equation over a range of 4·0–37·5°.

The writer is indebted to Mr P. S. Barnhart for the identification of the marine plants.

REFERENCES.

Marks (1934, 1). *J. Biol. Chem.* **105**, 489.
—— (1934, 2). *J. Biol. Chem.* **107**, 623.
—— and Fox (1933). *J. Biol. Chem.* **103**, 269.
Taylor (1930). A treatise on physical chemistry, 2nd ed., vol. **2**. (New York.)

LIX. THE EFFECT OF THYROTROPIC HORMONE UPON SERUM CHOLESTEROL.

By LEONARD IRVING PUGSLEY.

From the Department of Biochemistry, McGill University, Mon'real, Canada.

(*Received December 11th, 1934.*)

It has been shown by a number of workers that the administration of the thyrotropic hormone of the anterior pituitary has a stimulating effect upon the thyroid. The physiological properties and preparation of a highly active and purified extract containing this principle have been recently reported by Anderson and Collip [1934]. Hurxthal and his co-workers in a series of papers [1930; 1933; 1934] have shown that a reciprocal relationship exists between the blood cholesterol and the basal metabolic rate. Recently Cutting *et al.* [1934] have treated the published data on the relationship between basal metabolic rate and blood cholesterol statistically and have confirmed by a regression curve that an inverse relationship exists between these two variables. It has been shown experimentally by Westra and Kunde [1932] using rabbits, and Parhon and Derevici [1931] using dogs, that a decrease in blood cholesterol results from feeding desiccated thyroid and the injection of thyroxine.

In the light of this work, it seemed of interest to investigate the effect of the thyrotropic hormone upon serum cholesterol.

Methods.

A group of 15 adult rats weighing between 280 and 320 g. was chosen for the experiment. During the injection period, 3 or 4 rats were bled by heart puncture every second day. Successive samples were taken from each animal at 6-day intervals, in order to minimise the effects of haemorrhage. Between 1·2 and 1·5 ml. of blood were removed each time and 0·5 ml. of serum was used in the estimation of cholesterol, which was carried out by the method of Sackett [1925]. This amount of serum was found necessary on account of the low value for serum cholesterol in the rat. The normal level for cholesterol was obtained from a group of 12 rats and these were also bled at intervals of 6 days on different occasions to show that the removal of this quantity of blood did not interfere with the level of cholesterol.

The rats received twice daily 0·5 ml. of an active thyrotropic extract which was shown to contain 50 units of the hormone per ml. as assayed on the hypophysectomised rat.

Results.

The results are shown in Table I. The normal values for serum cholesterol of rats varied between 64 and 75 mg. per 100 ml. with an average value of 70 mg. per 100 ml. Upon injection of the thyrotropic hormone, the serum cholesterol decreased markedly, reaching its lowest level on the 8th to 10th day of injection and then returning to the normal level or slightly above in the course of 2 weeks. The values are expressed graphically in Fig. 1. This graph has a striking reciprocal relationship to the basal metabolic rate curves shown by Pugsley *et al.* [1934] in their study of the effect of thyrotropic hormone upon creatine and creatinine excretions of normal and hypophysectomised rats.

Table I. *The effect of thyrotropic hormone upon the serum cholesterol of rats.*

The values are expressed in mg. per 100 ml. of serum.
0·5 ml. b.i.d. Extract 2 intraperitoneally for 40 days.

Days	No. of determinations	Maximum	Minimum	Average
Control	12	75	64	70
2	4	60	57	59
4	4	60	49	54
6	4	64	48	59
8	4	64	64	64
10	3	55	40	46
12	6	60	43	49
14	4	58	53	54
16	3	54	51	53
18	4	74	60	66
20	4	74	52	66
22	3	77	54	68
24	4	80	66	75
26	4	95	71	82
30	3	96	69	81
32	3	95	66	83
34	3	74	68	72
36	3	83	64	74
40	4	87	63	73

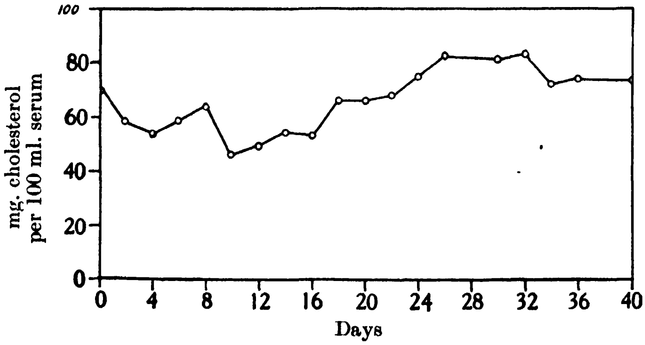

Fig. 1. The effect of thyrotropic hormone upon the serum cholesterol of rats.

The serum cholesterol has also been studied in dogs after intravenous and intraperitoneal injections of the thyrotropic hormone. The results are shown in Table II. In dog no. 1 it is seen that the injection of a potent thyrotropic extract caused a marked decrease in the serum cholesterol. This is confirmed in dog no. 2, using the extract employed in the experiments on rats. When the injections were discontinued the cholesterol values returned to the control level within 2 weeks. With continued injections, as is shown in dog no. 1 B, the same decrease in cholesterol was found, but the values did not return to the control level as rapidly as they did in the chronically-treated rats. The injections were continued for 68 days and the serum cholesterol was determined every second day; during this time the values ranged between 125 and 145 mg. per 100 ml. In dog no. 1 C it is shown that boiling the thyrotropic hormone at p_H 6·0 for 30 minutes in an open flask completely destroys its effect in lowering the serum cholesterol.

Table II. *The effect of thyrotropic hormone upon the serum cholesterol of dogs.*

The values are expressed in mg. per 100 ml. of serum.

Days	Dog no. 1	Dog. no. 2	Dog no. 1 B	Dog no. 1 C	Dog no. 3
Control	340	218	275	273	240
Control	342	220	272	270	240
2	333	218	208	270	240
4	253	160	168	274	240
6	200	155	135	265	218
8	171	137	120	272	222
10	163	130	114	—	220
12	—	120	114	—	222
16	—	125	100	—	218
20	—	177	107	—	240
24	273	218	145	—	—
28	—	—	141	—	—
38	—	—	133	—	—
48	—	—	130	—	—
68	—	—	123	—	—

Dog no. 1. Male. Wt. 16 kg. 10 ml. b.i.d. Extract 1 intravenously for 10 days.
Dog no. 2. Male. Wt. 15 kg. 5 ml. b.i.d. Extract 2 intraperitoneally for 12 days.
Dog no. 1 B. Male. Wt. 16 kg. 5 ml. b.i.d. Extract 2 intravenously for 68 days.
Dog no. 1 C. Male. Wt. 16 kg. 10 ml. b.i.d. Extract 1 intravenously (boiled) for 8 days.
Dog no. 3. Female. Thyroparathyroidectomised-hypophysectomised. Wt. 12 kg. 5 ml. b.i.d. Extract 2 intraperitoneally for 20 days.

The effect of the thyrotropic hormone upon the serum cholesterol of a thyroparathyroidectomised-hypophysectomised dog has been studied (dog no. 3). The completeness of these operations has not been established as yet. However, the thyroparathyroidectomy appears to be complete, since the serum calcium showed a marked decrease following the operation (11·6 to 7·8 mg. per 100 ml.). The animal received parathyroid hormone (Eli Lilly and Company) for some time, but during the administration of the thyrotropic hormone no parathyroid hormone was given. The dog was in good health and showed no symptoms of tetany during this time. The serum calcium which was also followed ranged between 7·0 and 8·0 mg. per 100 ml. In this dog no significant decrease in the cholesterol was observed as a result of the injections. This observation is in agreement with the reports of Verzar and Wahl [1931] and others that the thyrotropic hormone does not function in thyroidectomised animals.

SUMMARY.

The intraperitoneal injection of the thyrotropic hormone caused a marked decrease in the serum cholesterol of rats and dogs.

The serum cholesterol curve shows a reciprocal relationship to the basal metabolic rate curve of rats receiving chronic injections of the thyrotropic hormone.

I wish to express my indebtedness to Prof. J. B. Collip and to Dr D. L. Thomson for advice during the course of this work.

REFERENCES.

Anderson and Collip (1934). *J. Physiol.* **82**, 11.
Cutting, Rytand and Tainter (1934). *J. Clin. Invest.* **13**, 547.
Hurxthal (1933). *Arch. Int. Med.* **51**, 22.
—— (1934). *Arch. Int. Med.* **52**, 762.
Mason, Hunt and Hurxthal (1930). *New England J. Med.* **203**, 1273.
Parhon and Derevici (1931). *Compt. Rend. Soc. Biol.* **107**, 388.
Pugsley, Anderson and Collip (1934). *Biochem. J.* **28**, 1135.
Sackett (1925). *J. Biol. Chem.* **64**, 203.
Verzar and Wahl (1931). *Biochem. Z.* **240**, 37.
Westra and Kunde (1932). *Proc. Soc. Exp. Biol. Med.* **29**, 677.

LX. STUDIES ON THE PHOSPHATASES.

THE INFLUENCE OF SOME ELECTROLYTES ON THE PHOSPHATASES OF ANIMAL TISSUE. PHOSPHATASES OF THE LIVER, KIDNEY, SERUM AND BONES OF THE RABBIT.

By SERAFINO BELFANTI, ANGELO CONTARDI and ALBERTO ERCOLI.

From the Istituto Sieroterapico Milanese and R. Istituto Superiore Agrario, Milan.

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In recent work Davies [1934] found that the autolysed extracts of liver and spleen hydrolyse sodium β-glycerophosphate at two different optima of acidity, namely p_H 4·5–5 and p_H 8·9, and he expressed the opinion that there were two different phosphatases: however, it was not possible for him to purify the extracts in such a way as to obtain from them two fractions each containing one of the two distinct enzymes.

An analogous fact had been observed for the phosphatases of hog kidney by Kurata [1931] who had succeeded in obtaining, by adsorption processes and from the same starting material, two different enzymic solutions, one having an optimum of phosphatase activity at p_H 3, the other at p_H 9.

Recently some Japanese authors of Akamatsu's school [Usawa, 1932: Munemura, 1933, *etc.*] came to the conclusion that the enzymes which hydrolyse the monoesters of phosphoric acid belong to three types:

(*a*) A phosphomonoesterase type I, p_H optimum 3, extracted by Manaka [1931] from takadiastase.

(*b*) A phosphomonoesterase type II, p_H optimum 5·4–5·6, extracted by Usawa [1932] from rice husk and purified by an adsorption process.

(*c*) A phosphomonoesterase type III, p_H optimum 9–10, studied by Asakawa [1928; 1929] in kidney extracts.

Hori [1932], confirming the results obtained by Roche [1931], reported the presence of a phosphomonoesterase of type II in erythrocytes. In this way, according to the Japanese authors quoted, phosphomonoesterases of all three types are to be found in animal tissues, whereas type III phosphomonoesterase would appear always to be absent from vegetable tissues.

A study of the behaviour of the three phosphomonoesterases in the presence of different electrolytes has been made by Munemura [1933], who made his experiments on purified solutions of extracts of takadiastase, of rice husks and of hog kidney, chosen as being representative of the three types of phosphomonoesterase. Starting from the presupposition that all the enzymes which hydrolyse the monoesters of phosphoric acid and which exist in nature are included in these three types, he generalised the results thus obtained.

We wished to study the influence of some inactivating agents and, in particular, of sodium fluoride and oxalate, on the phosphatase activity of extracts of liver, kidney, bone and serum of the rabbit. It seems to us that the results we obtained lead to deductions which are somewhat different from those arrived at by the Japanese authors.

EXPERIMENTAL.

Technique.

The general method of research was as follows. In a series of 25 ml. flasks were placed: 1 ml. of a solution of sodium glycerophosphate (5 % of the anhydrous salt); 2·5 ml. of a solution of sodium acetate and sodium veronal in concentration double that prescribed by Michaelis [1930], containing 38·856 g. of $CH_3COONa, 3H_2O$ and 58·856 g. of sodium veronal per litre of solution; 2 ml. of an 8·5 % solution of pure NaCl. To each of the flasks was added, in order to obtain the desired p_H value, that quantity of HCl prescribed by Michaelis for the purpose; when it was desired to examine the action of some inhibiting agent a measured quantity of a concentrated solution of that agent was also added. Finally, 5 ml. of the enzymic solution under examination were placed in each flask and the liquid brought to the mark. In view of the fact that the enzymic extracts, like the solutions of oxalate and of fluoride, often notably displace the p_H of the buffer solutions, the p_H was controlled electrometrically on an aliquot part of the solution. The remainder was distributed in portions of 5 or 10 ml. in thick-walled test-tubes which, after the addition of a drop of toluene, were tightly closed and left in the thermostat at 37° for 16 hours. At the end of that time, a measured quantity of 10 % trichloroacetic acid was added to each test-tube. The contents were filtered and inorganic phosphorus was determined in the filtrate using Lohmann and Jendrassik's [1926] modification of Fiske and Subbarow's [1925] method. The values thus ascertained were then calculated to 1 ml. of the solution under examination.

We used the acetate-veronal buffer according to Michaelis, notwithstanding its weak buffering powers, because it offers the advantage of an extremely wide range of acidity, between p_H 9·64 and 2·62, simply by adding different amounts of hydrochloric acid. When it was necessary to use still higher p_H values (10 to 11), we added a few drops of dilute solution of sodium hydroxide to the veronal buffer, counting also on the buffering power of the enzymic solutions themselves. In this way we excluded the possibility of the enzymes being diversely influenced by the different nature of the buffer.

The crystallised sodium glycerophosphate used by us, and placed on the market by Messrs Carlo Erba of Milan, was composed exclusively of the β-form [Carrara, 1932].

Phosphatases of liver and kidney extracts.

The tissues used in these experiments were taken from adult and normal rabbits. The technique employed for the preparation of the extracts had many points of similarity with that adopted by Asakawa [1928]. The animals were killed by cutting the carotid. The abdominal cavity was then immediately opened, a cannula needle was introduced into the vena cava and, after ligaturing the portal vein, the blood vessels were washed with physiological salt solution at 37°. The organs were then removed and, after being freed from the residues of surrounding tissues, were rapidly washed with physiological salt solution, weighed, triturated with sand in a mortar and placed in an Erlenmeyer flask together with ten times their weight of sterile distilled water and a few ml. of toluene. The whole was then placed in a thermostat at 37° and allowed to autolyse for 48 hours. It was afterwards filtered through a folded filter. The clear filtrate was dialysed for 36 hours in small bags of collodion placed in distilled water. The dialysed extracts were again filtered, a small quantity of toluene was added and the whole kept in an ice-chest. The extracts thus prepared do not contain enzymes from the blood when the operation of washing with physiological salt solution has been conducted properly. They are, moreover, extremely poor in albuminous substances and keep their phosphatase activity for a fair length of time. Again, possible sources of error, including those due to the hydrolytic action of phosphodiesterases and pyrophosphatases, are avoided because the phosphorylated organic compounds pre-existing in the tissues are decomposed during the digestion in the thermostat and the inorganic phosphates are eliminated wholly by means of the dialysis. The determinations themselves thus become less laborious because controls are not necessary.

Table I shows that, within very wide limits, the effect of sodium fluoride on the phosphatase activity of a liver extract does not change with variations in the degree of concentration of this salt.

Table I. *Inhibitory action of sodium fluoride on the phosphatase activity of rabbit liver extracts.*

p_H of the buffer solution	mg. P hydrolysed in the presence of NaF				mg. P hydrolysed in the absence of NaF
	$M/50$	$M/7$	$M/4$	$M/2$	
3·6	—	0·006	—	—	0·034
5·5	0·003	—	0·005	0·007	0·067
7·5	0·040	—	0·039	0·040	0·050
8·3	0·067	0·066	0·067	0·065	0·065

From Fig. 1, which represents the results of an experiment carried out by acting contemporaneously with an extract of rabbit liver on sodium glycerophosphate in the absence of inhibitory agents (Curve A), in the presence of $M/50$ NaF (Curve B), and in the presence of sodium $M/50$ oxalate (Curve C), it may be seen that, analogously with the results obtained by Davies [1934], the

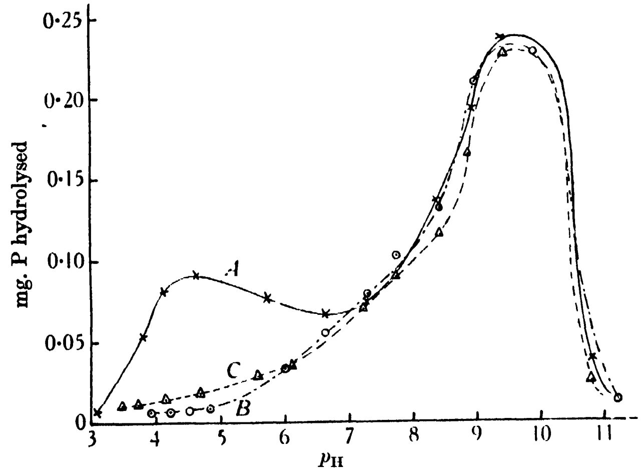

Fig. 1. The hydrolysis of β-glycerophosphate in the absence and presence of added fluoride and oxalate by rabbit liver extracts under varying conditions of hydrogen ion concentration.

Curve A. × ——— × Hydrolysis in absence of inactivating agents.
Curve B. ⊙ — — —⊙ Hydrolysis in presence of added fluoride.
Curve C. ▲- - - - ▲ Hydrolysis in presence of added oxalate.

phosphatase of the liver acts at two different p_H optima, one in an alkaline zone and the other in an acid zone. It is also clearly shown that the two inhibitory agents examined leave the "alkaline" phosphatase activity almost unaltered and that they affect only the "acid" phosphatase activity. As this inactivation is very profound and the residual activity from p_H 3 to 6 is almost negligible, it may be held that the problem set by Davies [1934] to separate the two phosphatases has been solved at least in part.

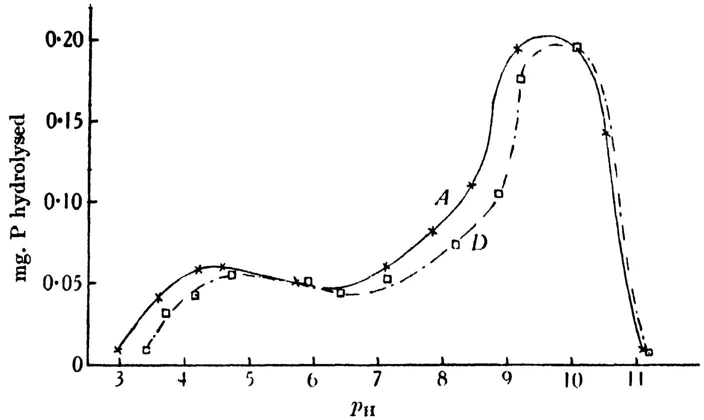

Fig. 2. The hydrolysis of β-glycerophosphate in the absence and presence of added iodoacetate by liver extracts (rabbit) under varying conditions of hydrogen ion concentration.

Curve *A*. ×——× Hydrolysis in absence of added iodoacetate.
Curve *D*. ⊡—·—·⊡ Hydrolysis in presence of added iodoacetate.

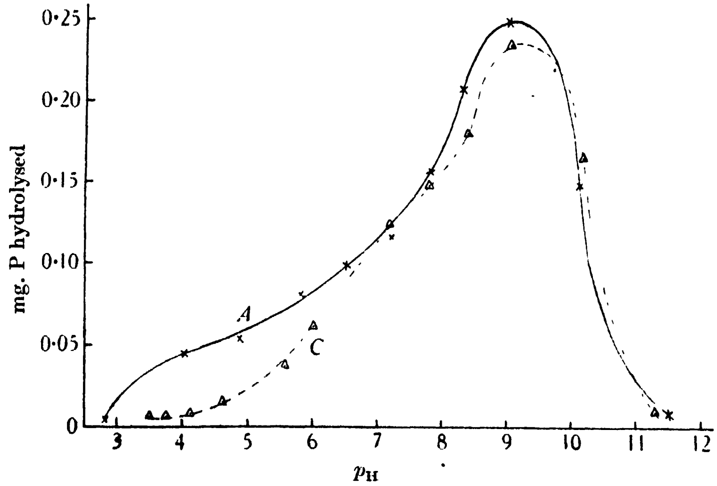

Fig. 3. The hydrolysis of β-glycerophosphate in the absence and presence of added oxalate by kidney extracts (rabbit) under varying conditions of hydrogen ion concentration.

Curve *A*. ×——× Hydrolysis in absence of added oxalate.
Curve *C*. △----△ Hydrolysis in presence of added oxalate.

The results of this experiment are not in agreement with the views of some of the Japanese authors mentioned above. In fact, according to Munemura [1933], sodium oxalate completely inhibits the activity of the phosphatase type

III (alkaline) whereas this did not occur in our case. Again, according to the same author the oxalate should not have a very noticeable action on type II phosphatase at a p_H optimum of 5·5. It should be observed, however, that, in analogy with what was found by Kobayashi [1927] and Inouye [1928] in the case of the phosphatase of *A. oryzae*, our acid phosphatase might also be a type I phosphatase modified in its properties by the presence of a substance *X*.

Table II. *Inhibitory action of sodium trichloroacetate on the phosphatase activity on extracts of rabbit liver.*

p_H of the buffer solution	mg. P hydrolysed		
	In the presence of CCl_3COONa		In the absence of CCl_3COONa
	M/50	*M*/20	
3·2	0·0089	0·0067	0·0338
5·3	0·0674	0·0686	0·0649
8·9	0·0828	0·0805	0·0828

From Table II it may be seen that sodium trichloroacetate has a similar action to those of sodium fluoride and oxalate on the phosphoesterase of the liver.

Fig. 2 shows the behaviour of the same extract of liver on sodium β-glycerophosphate in the presence and in the absence of *M*/50 sodium iodoacetate. This liver extract was the same as that used for the experiment illustrated in Fig. 1. The experiment was, however, carried out 6 days later with the same enzymic solution which had been kept in the ice-chest in the meantime. As may be seen, whilst the "alkaline" phosphatase activity remained almost unaltered, the "acid" phosphatase underwent a much more noteworthy inactivation. The iodoacetate acts extremely weakly on the acid phosphatase, and it appears to have only the same weak influence on the alkaline phosphatase as have oxalate and fluoride.. The determinations of the p_H in the presence of iodoacetate under alkaline conditions were carried out by means of the colorimeter because the iodoacetate poisons the hydrogen electrode.

Fig. 3 shows the action of the phosphatase of a rabbit kidney extract on β-glycerophosphate in the absence (Curve *A*) and in the presence (Curve *C*) of *M*/50 sodium oxalate. In Curve *A*, besides the optimum of activity at p_H 9·2, the existence of a second optimum in the acid zone is not to be found. However, in the presence of sodium oxalate under acid conditions there is a marked inhibition of the phosphatase activity, analogous to that which occurs in the case of liver extracts. Therefore, in this case also one may suppose the presence of an "acid" phosphatase. The considerations already set forth in the matter of the "acid" phosphatase of the liver hold good also in this case and are supported by the experiments, of which we have already made mention, carried out by Kurata [1931] on the phosphatases of hog kidney.

As regards the "alkaline" phosphatase, we were not able to find inactivation by sodium oxalate in extracts of rabbit kidney prepared by the method used by Asakawa and by Munemura. This is the contrary of what Munemura found when experimenting with extracts of hog kidney.

On the whole, the behaviour of the phosphatases of the liver and those of the kidney, in the presence of inactivating agents, is such as to cause one to admit the qualitative identity of the two enzymic systems.

Phosphatases of rabbit and horse blood serum.

We used the serum from 8 rabbits which were kept without food for 24 hours. The blood was taken from the carotid. The horse serum was obtained from an animal which had been kept without food for 24 hours. In this case, the blood was taken by opening the external jugular vein.

The sera thus obtained were dialysed directly, without predigestion in the thermostat, in small bags of collodion placed in distilled water for 36 hours. Fig. 4 shows the hydrolysis of β-glycerophosphate produced at different p_H values in the absence of inhibitory agents (Curve *A*), in the presence of *M*/50 NaF (Curve *B*), in the presence of *M*/50 sodium oxalate (Curve *C*) and in the presence of *M*/100 sodium iodoacetate (Curve *D*).

Fig. 5 shows the hydrolysis produced by dialysed horse serum in the absence of inhibitory agents (Curve *A*), in the presence of *M*/50 NaF (Curve *B*), and in the presence of *M*/50 sodium oxalate (Curve *C*).

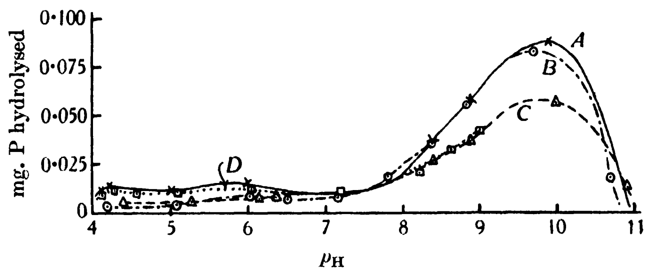

Fig. 4. The hydrolysis of β-glycerophosphate in the absence and presence of added fluoride, oxalate and iodoacetate by rabbit serum under varying conditions of hydrogen ion concentration.

Curve *A*. × ——— × Hydrolysis in absence of inactivating agents.
Curve *B*. ⊙—·—·⊙ Hydrolysis in presence of added fluoride.
Curve *C*. △- - - -△ Hydrolysis in presence of added oxalate.
Curve *D*. ▣ · · · · · ▣ Hydrolysis in presence of added iodoacetate.

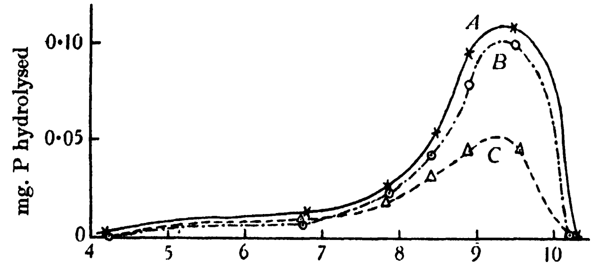

Fig. 5. The hydrolysis of β-glycerophosphate in the absence and presence of added fluoride and oxalate by horse serum under varying conditions of hydrogen ion concentration.

Curve *A*. × ——— × Hydrolysis in absence of inactivating agents.
Curve *B*. ⊙—·—·⊙ Hydrolysis in presence of added fluoride.
Curve *C*. △- - - -△ Hydrolysis in presence of added oxalate.

In serum one also observes the presence of an acid phosphatase, although very weak. In the presence of inhibitory agents, it behaves in the same way as the extracts of liver and kidney. As regards the alkaline phosphatase, this is not inactivated by sodium fluoride but is greatly inhibited by the action of sodium

oxalate. As in the case of the liver extract, iodoacetate does not much affect the acid phosphatase; it acts on the alkaline phosphatase, at least up to the p_H values employed in the same way as sodium oxalate.

The phosphatases of bone.

The femora, tibiae and humeri of a 25-day old rabbit were carefully freed from their soft parts. The epiphyses were detached, the bones split along their

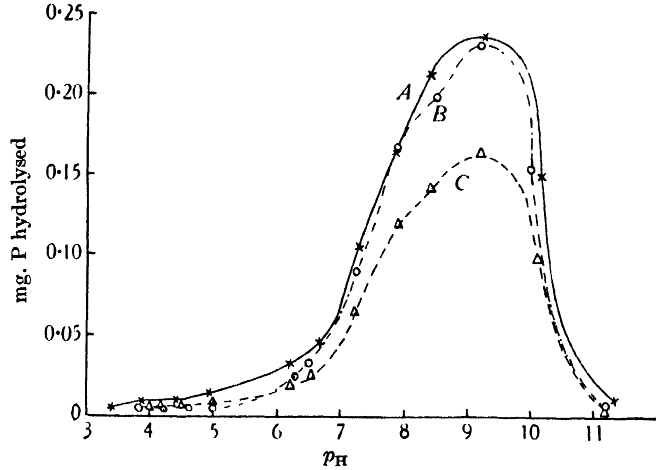

Fig. 6. The hydrolysis of β-glycerophosphate in the absence and presence of added fluoride and oxalate by rabbit bone (shaft) extracts under varying conditions of hydrogen ion concentration.
Curve A. ×——× Hydrolysis in absence of inactivating agents.
Curve B. ⊙-·-·⊙ Hydrolysis in presence of added fluoride.
Curve C. △- - - -△ Hydrolysis in presence of added oxalate.

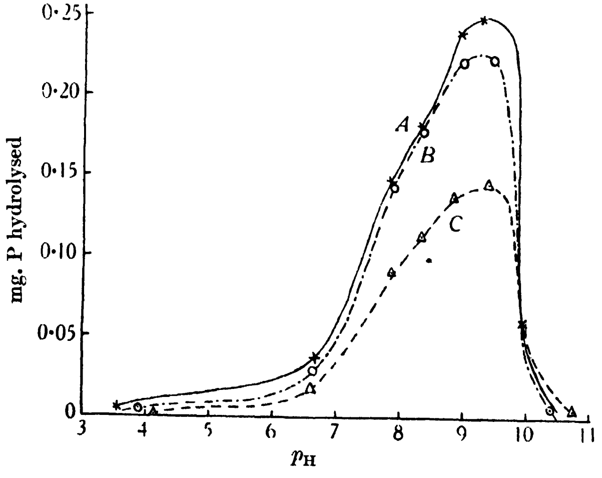

Fig. 7. The hydrolysis of β-glycerophosphate in the absence and presence of added fluoride and oxalate by rabbit bone (epiphysis) phosphatase under varying conditions of hydrogen ion concentration.

Curve A. ×——× Hydrolysis in absence of inactivating agents.
Curve B. ⊙—·—·⊙ Hydrolysis in presence of added fluoride.
Curve C. △- - - -△ Hydrolysis in presence of added oxalate.

length and the marrow carefully removed. They were then rapidly washed with physiological salt solution. The shafts and the epiphyses, after having been broken up separately in a mortar with quartz sand, were subjected to the treatment described in connection with the extracts of kidney and liver.

Figs. 6 and 7 show the hydrolyses produced respectively by the extracts of shaft and of epiphysis at various degrees of acidity in the absence and in the presence of fluoride and of oxalate. As may be seen, the two extracts behave in an analogous manner.

Examination of the two diagrams shows that the considerations set forth in the case of the phosphatases of serum may be repeated in the case of bone. The two systems behave in a markedly similar manner towards oxalate and fluoride. In young animals the phosphatase of the bone is much more active than that of the serum.

DISCUSSION.

Examination of the diagrams previously explained shows the following facts.

(1) Extracts of liver and kidney contain, besides the alkaline phosphatase which is that which up to the present has most attracted the attention of research workers, an "acid" phosphatase which has some activity but,in any case is not negligible. It seems probable to us that this "acid" phosphatase has a physiological function.

(2) In bones (shaft and epiphyses) and in blood serum, the activity of the "acid" phosphatase is extremely slight.

(3) In extracts of bone and in serum, the "alkaline" phosphatase is sharply differentiated from the corresponding one present in extracts of liver and kidney by the fact that sodium oxalate reduces its activity in greater measure.

(4) Near neutrality, the influence of the inactivating agents which we have considered is negligible. One might therefore distinguish three different systems of phosphatase enzymes in animal tissues: that of the liver and of the kidney (and probably also of the spleen [Davies, 1934]) characterised by the presence of an "acid" and an "alkaline" phosphatase, the latter not being inhibited by sodium oxalate; that of serum and of bone characterised (the acid phosphatase being nearly negligible) by the presence of an "alkaline" phosphatase which is partially inhibited by sodium oxalate;[1] and lastly, that of the erythrocytes characterised, according to the statements of Roche [1931] and Hori [1932], by the presence of the acid phosphatase only. Moreover, according to Hori, this phosphatase of the erythrocytes (p_H optimum 5·5) is different from that of the kidney (p_H optimum 3).

In order to explain the negligible activity of the "acid" phosphatase of bone extract, one might put forward the hypothesis that the accumulation in the bones of notable quantities of fluoride which, as we have seen, inhibits the activity of this phosphatase, contributes to this fact.

We are working on the influence of fluorides on phosphatases; the results of our experiments will be given in one of our next communications.

We believe in fact that fluorine plays an important part in the phenomena of calcification and decalcification of bone and that by carrying research work still farther in this direction, it may be possible to obtain some light on this complicated question.

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[1] We wish to observe in this connection that most authors, on the basis of Kay's work [1930], hold that the phosphatase of serum is of osseous origin: latterly however the work of Bodansky [1934] and of Bodansky and Jaffe [1934] tends to show that, except in cases of disease of the bone, the phosphatase of serum is of different origin.

Many considerations confirm this point of view.

(*a*) The fixation of fluorine by the bone, a phenomenon which in itself must surely have some physiological meaning.

(*b*) The phenomena consequent on chronic fluoride poisoning. These phenomena, on which a vast literature already exists [McClure, 1933], may easily be associated with a degenerated phosphatase activity of the osseous tissues.

(*c*) The alterations of the phosphatase activity of the plasma recently observed by Phillips [1934] in cows suffering from fluorosis. In this connection we have already referred to the analogy which exists between the phosphatase system of serum and that of bone.

(*d*) The fact, discovered by Robison and Rosenheim [1934],[1] that the addition of sodium fluoride, even in extremely small quantities, impedes *in vitro* the deposition of calcium on the ossifying cartilage.

To these considerations it may be objected that, in all the cases observed up to the present, the fluoride acts only on the acid phosphatases and not on the alkaline ones which appear, as regards the process of calcification of the bones and having regard to the reaction of the media in which they must operate, to be the most important. Robison and Rosenheim [1934] have observed that fluoride does not depress the activity of the bone phosphatases (alkaline). Furthermore, it may be objected that, in the phenomena of calcification, the two mechanisms admitted by Robison (scission of the phosphoric ester and precipitation of the calcium phosphate) may occur even at different hydrogen ion concentrations. Experiments not yet completed demonstrate that in certain conditions fluorides may also inhibit the activity of the alkaline phosphatases.

At this point we wish to recall attention to the classification of the phosphatases proposed by the Japanese authors. It has already been said that Akamatsu and his collaborators distinguish three types of phosphomonoesterase corresponding to the three different degrees of dissociation of a monoester of phosphoric acid (the hydrogen of both hydroxyls not replaced, p_H 2·8–3; that of one hydroxyl replaced, p_H 5·5; that of both hydroxyls replaced, p_H 9–10) and analogous to the three types of proteinase: pepsin, papain and trypsin-kinase. From several points of view, this classification appears to be most suggestive, but it is difficult to accept entirely the views of the Japanese school on the behaviour of these three types of phosphatase as regards inhibitors and chemical activators. For example, we have seen how, according to Munemura [1933], the activity of the alkaline phosphatase should be completely inhibited in the presence of sodium oxalate. We found instead, in some of the cases examined, only partial inactivation, whilst in other cases the inactivation was practically nil. From this, ought one to deduce that there exists a fourth type of phosphatase having an optimum of activity at the same p_H as the third type, but which can be distinguished from the latter because it maintains its activity even in the presence of sodium oxalate? It appears to us that it is more probable that the inactivation of the enzymes by inhibitory agents as commonly used may be exerted, at least in certain cases, not on the haptophore groups but on the colloidal carriers of the enzyme itself. In these cases, therefore, one and the same enzyme changes its properties towards activating and inactivating agents when there are changes in the colloidal carrier. Objections might be raised to the technique employed by us because the determinations were made after a somewhat lengthy period of digestion in the thermostat (16 hours) so that the hydrolysis of the substrate, at the optimum p_H, in some cases reached 80 %. Moreover, the experiments were made with a veronal buffer. The determinations carried out by Munemura [1933], using a glycine buffer with an extract of hog kidney in the presence and in the absence of sodium oxalate, were made when the hydrolysis of the glycerophosphate, in the absence of oxalate and at the optimum p_H, was about 50 %. It may be suspected that this is the cause of the notable difference between our results and those of the Japanese author.

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[1] For the sake of brevity, we have omitted to cite the other works of Robison to whom, together with Kay, belongs the merit of having commenced and of having given great impulse to the study of the phosphatases. On the other hand, the works of both these authors and of their collaborators, published for the greater part in this *Journal* since 1923, are well known to all those who are interested in this subject.

Preliminary experiments have however shown us that the conclusions are not substantially changed either when the hydrolysis is interrupted at 50 % or when it is still further prolonged, and this both in the presence of veronal buffer and in that of glycine buffer.

This preliminary examination led, among other things, to interesting observations on which we will report in our next communication.

On the other hand, when carrying out the inorganic phosphorus determinations we found it possible to obtain, after a sufficiently long space of time, detectable quantities of phosphorus even at acid p_H values and thus to have the possibility of a simultaneous and accurate measure of the activities of both acid and alkaline phosphatases.

We feel that it is opportune to make a further remark in connection with the use of sodium oxalate as an anticoagulant for the taking of blood for research work on the phosphatases of the plasma. It is clear, from what we have explained, that by operating in this manner, the action of the phosphatases present is greatly inhibited. Bodansky [1933] in fact observed that the phosphatase activity of oxalated plasma was about 10 % lower than that of heparin plasma. He, however, attributed this to the increase in osmotic pressure due to the potassium oxalate and to the consequent dilution of the plasma.

For the same reasons it is inadvisable to employ trichloroacetate which some authors have used when studying the scissions of the phosphorylated compounds of the blood.

SUMMARY.

1. Extracts of liver and kidney contain, besides an "alkaline" phosphatase, an "acid" phosphatase, the activity of which is not negligible.

2. The activity of the "acid" phosphatase of liver and kidney extracts is inhibited by the fluoride, oxalate and trichloroacetate of sodium, that of the "alkaline" phosphatase of the same extracts is not influenced by these electrolytes. In this connection the two enzymic systems are qualitatively similar.

3. The activity of the "acid" phosphatase is extremely low in bone and serum.

4. In serum and extracts of bone, the "alkaline" phosphatase is partially inhibited by sodium oxalate. From this point of view these two enzymic systems may be considered to be qualitatively identical.

5. Near neutrality the influence of the inactivating agents which we have considered is negligible.

6. The influence of fluorides on the processes of calcification and decalcification of bone is discussed.

REFERENCES.

Asakawa (1928). *J. Biochem.* **9,** 134.
—— (1929). *J. Biochem.* **10,** 157.
Bodansky (1933). *J. Biol. Chem.* **101,** 93.
—— (1934). *Arch. Int. Med.* **54,** 88.
—— and Jaffe (1934). *J. Biol. Chem.* **104,** 473.
Carrara (1932). *Giorn. Chim. Ind. Appl.* **14,** 236.
Davies (1934). *Biochem. J.* **28,** 529.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66,** 375.
Hori (1932). *J. Biochem.* **16,** 433.
Inouye (1928). *J. Biochem.* **10,** 133.
Kay (1930). *J. Biol. Chem.* **89,** 249.
Kobayashi (1927). *J. Biochem.* **8,** 205.
Kurata (1931). *J. Biochem.* **14,** 42.
Lohmann and Jendrassik (1926). *Biochem. Z.* **178,** 519.
Manaka (1931). *J. Biochem.* **14,** 191.
McClure (1933). *Physiol. Rev.* **13,** 277.
Michaelis (1930). *Biochem. Z.* **234,** 140.
Munemura (1933). *J. Biochem.* **17,** 343.
Phillips (1934). *Science,* **76,** 239 (New York).
Robison and Rosenheim (1934). *Biochem. J.* **28,** 684.
Roche (1931). *Biochem. J.* **25,** 1724.
Usawa (1932). *J. Biochem.* **15,** 1.

LXI. STUDIES ON THE ANAEROBIC DECOMPOSITION OF PLANT MATERIALS.

I. THE ANAEROBIC DECOMPOSITION OF RICE STRAW (*ORYZA SATIVA*).

BY CADAMBI NARASIMHA ACHARYA.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden, Herts.

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THE aerobic decomposition of plant materials has in recent years attracted much attention, on account of its importance in the formation of synthetic manure, in the decomposition of soil organic matter, in the purification of sewage and trade wastes, in the decay of wood *etc.*, but comparatively little is known regarding the course and rate of decomposition under anaerobic and partially aerobic conditions. Tenney and Waksman [1930] have reported some data in this connection and a certain amount of work has been done in regard to the changes in organic matter in marshy and peaty soils, generally associated with such conditions. While the agricultural importance of such soils is comparatively limited, in other cases anaerobic and semi-aerobic conditions have played a significant rôle in increasing crop production and have been objects specially aimed at in the system of cultivation, *e.g.* in the production of rice under swamp or water-logged conditions as practised in most parts of India and China and to an increasing extent in Burma. The biochemistry of the changes in organic matter and other constituents in such soils has been examined by Nagaoka [1905], Daikuhara and Imaseki [1907], Harrison and Iyer [1913; 1914; 1915; 1916; 1919; 1920], Panganiban [1923], Subrahmanyan [1927; 1929; Subrahmanyan and Sreenivasan, 1934], Itano and Arakawa [1927; 1932], Metzger [1929; Metzger and Janssen, 1928], Joachim and Kandiah [1929], Misu and Shimohira [1929], Bartholomew [1929], Robinson [1930], Osugi *et al.* [1931; 1932] and others. But their work has been done mostly under the semi-aerobic conditions of the water-logged soil and no detailed study of the decomposition of organic matter under strictly anaerobic conditions has been reported. Data under such conditions, it was thought, might serve not only for purposes of comparison, but might also help in the interpretation of the results obtained already under water-logged conditions.

The object of the experiments reported in this and succeeding papers was to examine in detail the course of anaerobic decomposition of a variety of plant materials by themselves and in admixture with soil, as compared with the course of aerobic decomposition on the one hand and with the decomposition under water-logged or swamp soil conditions, on the other.

Recently much work has been reported on the anaerobic digestion of sewage and trade wastes, with special reference to securing the maximum evolution of combustible gases [*cf.* Whitehead and O'Shaughnessy, 1931; Adeney, 1932; Heukelekian, 1933; Rudolfs, 1932; Rudolfs and Heukelekian, 1932; Rudolfs and Setter, 1932; Buswell and Boruff, 1932; Larson *et al.* 1934]. But it should be noted that the conditions influencing the decomposition of trade wastes or sewage, where the concentration of the dissolved or suspended matter is comparatively low, may vary markedly from those controlling the decomposition of plant materials in much higher concentrations and in presence of soil. Comparisons of data obtained under the two sets of conditions may, however, be made with advantage and will be discussed later.

A different aim has guided several workers [Richards and Amoore, 1920; Fowler and Joshie, 1920; Langwell and Lymn, 1923; 1932; Boruff and Buswell, 1929], who have examined the anaerobic or semi-aerobic decomposition of plant materials on the technical scale with a view to obtaining the maximum yield of gaseous products. As their results bear close comparison with some data obtained during the course of the present experiments, their work may be considered in somewhat greater detail. Fowler and Joshie [1920] fermented various cellulosic materials and found that maximum gas evolution occurred at 30°, the mixed gases evolved having an average composition of 80–90 % CH_4, 4–12 % H_2 and 3–7 % CO_2. Hemicelluloses gave maximum gas production, and an acidity of more than 1 % retarded further fermentation. Langwell and Lymn [1923; 1932] adopted thermophilic digestion at 60° and observed that celluloses from different sources, hemicelluloses, starches and sugars could be easily fermented while the so-called ligno- and cuto-celluloses were unfermentable. Cereal straws were intermediate between true and ligno-celluloses and left an unfermentable residue of cellulose, apparently in combination. The addition of phosphorus and nitrogen was found to promote the fermentation. Aeration of the medium increased the amount of ethyl alcohol produced and a portion of the methane was replaced by hydrogen. Richards and Amoore [1920] working with straw and Nile sudd found that the optimum conditions for the production of methane were (1) a temperature between 35 and 40°; (2) exclusion of air; (3) ample water supply; (4) presence of some available nitrogen compound and (5) absence of acidity, which could be secured by addition of $CaCO_3$.

The methane fermentation of simple organic compounds and of sewage sludge has been followed by Buswell and co-workers [Buswell and Boruff, 1932; Symons and Buswell, 1933; Tarvin and Buswell, 1934; Boruff and Buswell, 1934]. This type of fermentation is held to be an oxidation-reduction reaction under anaerobic conditions, catalysed by bacteria and resulting in the production of almost theoretical quantities of CH_4 and CO_2: the primary products appear to be CO_2 and H_2, which react to form methane. Decarboxylation, deamination and hydrolysis were the principal means of decomposition, while the lower fatty acids were the chief intermediate compounds produced. A larger weight of substance was found as gas than was decomposed as organic matter, owing to the water present taking part in the reactions.

TECHNIQUE.

Rice straw from the Government Central Farm, Coimbatore, India, was chaffed into pieces about an inch long, and weighed quantities were packed into bottles of known volume fitted with rubber stoppers carrying exit tubes and pinch-cocks. Mineral nutrients, soil inoculant and distilled water in the quantities specified in the experiments described below were added, the contents well mixed and the stoppers secured by wiring with copper wire and then coating with paraffin wax. The enclosed air was removed by suction and the bottles were incubated at the required temperature. At specified intervals, generally once a week, the accumulated gases were tapped off through the exit tube into a Hempel's burette, measured and analysed for CO_2, CH_4, H_2 and N_2. At the end of the period of incubation, usually determined by the stoppage of gas evolution, or at intermediate intervals, samples were opened and the contents analysed. The liquid portion was separated from the solid matter by filtration through a

Büchner funnel and washing. The residue was dried, weighed and analysed for cellulose, lignin, furfuraldehyde-yielding compounds, protein and ash. The filtrate was made up to a known volume and aliquots were taken for the determination of volatile and non-volatile acids, total, ammoniacal, protein-, nitrate- and amino-nitrogen and carbonates.

Cellulose was determined by the method of Norman and Jenkins [1933]. Furfuraldehyde was estimated by Kröber's phloroglucide method, with final treatment of the phloroglucide with boiling alcohol to remove non-pentosan derivatives. The total furfuraldehyde yield and that obtained from the cellulose fraction are expressed in the accompanying tables in terms of xylan, for purposes of comparison of the hemicellulose fractions associated with cellulose and not so associated. It is realised that a portion of the total furfuraldehyde yield may be due to uronic acid derivatives, and that the hemicelluloses in rice straw may not all be in the form of xylan. Norman [1929] found that in oat and rye straws, the furfuraldehyde contributed by uronic acids was about 6–7 % of the total furfuraldehyde yield and it is probable that the correction necessary in the present case may be of the same order.

Lignin determinations were made by the 72 % sulphuric acid method (15 hours, below 15°) both on the material without any treatment (referred to in the tables as "lignin direct") and after hydrolysing it with 5 % sulphuric acid (referred to in the tables as "lignin after hydrolysis") [*cf.* Norman and Jenkins, 1933, p. 826]. It was found that the latter method gave uniformly lower results than the former, the difference varying from 10 to 30 % of the "lignin-direct" figure. In both cases, the ash content and nitrogen calculated as protein were deducted before arriving at the lignin figures. The allowance for nitrogen on the basis of its protein value is made in the absence of any definite knowledge of the form in which nitrogen is associated with lignin, though recent evidence [Norman and Jenkins, 1934] tends to show that the correction "factor" for nitrogen is liable to considerable variation depending on the nature of other substances present, especially furfuraldehyde-yielding compounds.

Volatile acids were estimated by steam-distillation, and the proportion of the constituents in the distillate was determined by the method of Virtanen and Pulkki [1928]. The fractional distillation constants obtained by this method for successive 25 ml. fractions agreed with the equations for a mixture of acetic and butyric acids. Qualitative tests on the distillate showed that it was almost wholly a mixture of acetic and butyric acids; there were only traces of formic, propionic and valeric acids.

Lactic acid was determined on the non-volatile liquid residue and, for comparison, also on an aliquot of the original solution, by the permanganate oxidation method of Friedmann and Graeser [1933]. Only negligible amounts of lactic acid could be detected at any stage.

Amino-nitrogen was estimated by Foreman's formaldehyde titration method as outlined by Brown [1923].

Determinations of the total, ammoniacal, nitrate- and protein-nitrogen were made by the methods recommended by the Association of Official Agricultural Chemists, U.S.A. [1930]. Volumes of gases have been given in the Tables after reduction to N.T.P.

EXPERIMENTAL.

Preliminary experiments showed that temperature and reaction exerted a profound influence on the rate and course of anaerobic fermentation. The extent of this influence and that due to other factors will be examined in detail in a later communication, but it may be noted here that a range of 30–35° and a slightly alkaline reaction (p_H 7·5–8·0) were found optimum for the progress of the decomposition. The maintenance of an alkaline reaction offered special difficulties on account of the large amount of organic acids formed rapidly in the early stages of the decomposition. The use of buffers at the high concentrations necessary to maintain constancy of the reaction, effectively inhibited bacterial activity as shown by absence of decomposition. Calcium carbonate, generally recommended in bacterial technique for use with gas-forming anaerobes was found (*cf.* Exp. 2 below) ineffective for maintaining a neutral reaction, especially in presence of CO_2 under pressures higher than atmospheric, and gas production was greatly inhibited even though there was excess of $CaCO_3$ present. It was found that the addition of a sufficient amount of ammonium carbonate (to the extent of 2 % nitrogen on the rice straw) served to maintain the p_H on the alkaline side. Sodium nitrate, when added to straw at the rate of 2 % nitrogen, also produced, after denitrification, sufficient alkali to neutralise the acids formed. In a few cases, however, the above proportion of sodium nitrate was found to result in too great an alkalinity in the early stages, while the amount of ammonium carbonate at 2 % nitrogen on straw taken was found insufficient to neutralise the acids formed; in these cases gas production was impeded. So in later experiments, attempts were made to maintain the p_H by an addition of other neutralising agents like $KHCO_3$, $NaHCO_3$, NH_4HCO_3 *etc.* and also by the addition of alkali at regular intervals. A discussion of these experiments will be reported later.

Exp. 1. Course of decomposition of rice straw without the addition of nitrogen or calcium carbonate. To 20 g. portions of rice straw were added 10 ml. of an aqueous extract of a garden soil (1 g. soil to 50 ml. water), 0·5 g. K_2HPO_4, 0·05 g. $MgSO_4, 7H_2O$ and enough distilled water to make up 200 ml. in all. The bottles were evacuated, sealed and incubated as described above. At the end of stated intervals one of the bottles was opened and the contents analysed. There was no gas evolution from any of the bottles though a certain amount of CO_2 was found in solution. The results of analysis, calculated per 100 g. original straw taken, are given in Table I.

It will be noted that the p_H of the medium falls at the end of the decomposition to 4·4. The accumulation of acids amounts to about 6·6 g. of which the major portion is acetic (about 4·0 g.) and the remainder butyric. About 20 % of the total hemicelluloses expressed as xylan, 8–9 % of cellulose and 5–7 % of lignin, as well as 35–37 % of the proteins, have been decomposed. The almost stationary state of the decomposition figures after the first month, during which most of the acid formation takes place (Table I), indicates that the progress of the decomposition is inhibited by the accumulation of acids and consequent lowering of p_H (*cf.* Exps. 4–6).

As regards nitrogen distribution, it is interesting to note that the major portion of the nitrogen which goes into solution remains in the form of protein and only a small portion is liberated as ammonia. Of the total nitrogen originally present in the straw, about 65 % remains in the solid residue, about 25 % is present in solution in the form of protein and only about 8–10 % is converted into ammonia. A similar kind of nitrogen distribution is also to be found in the succeeding experiments.

Exp. 2. Decomposition of rice straw without the addition of nitrogen but with $CaCO_3$ added as neutralising agent. To 20 g. portions of rice straw, 4 g. of $CaCO_3$ were added to neutralise the acids formed. The other experimental details were the same as in Exp. 1.

The results of analysis are shown in Table II. They show that in the presence

Table I. *Decomposition of rice straw without the addition of nitrogen or any neutralising agent.*

	100 g. original straw	After			
		1 month	2 months	3 months	4 months
	g.	g.	g.	g.	g.
		(A) Losses of constituents.			
Dry matter less ash	81·15	13·85	14·65	14·85	14·95
Straw cellulose (including xylan)	45·45	3·31	3·69	3·47	4·09
Xylan in cellulose	11·97	0·79	0·77	0·95	1·11
Xylan in polyuronides	9·50	3·90	3·01	3·71	3·01
Total xylans	21·47	4·69	3·78	4·66	4·11
Lignin (direct)	10·89	0·47	0·43	0·36	0·57
Lignin (after hydrolysis)	9·31	0·40	0·64	0·66	0·88
Protein	2·94	1·08	1·10	0·83	1·06
		(B) Products formed.			
CO_2 in solution	—	0·79	0·77	0·81	0·84
Acetic acid	—	3·35	3·97	4·07	3·75
Butyric acid	—	1·89	1·95	2·52	2·83
Total weight of products	—	6·03	6·69	7·40	7·42
p_H of medium	6·4	4·8	4·6	4·4	4·4
		(C) Nitrogen distribution.			
Initial N content	0·469	—	—	—	—
Protein-N in residue	—	0·296	0·294	0·335	0·299
Protein-N in aqueous extract	—	0·105	0·112	0·088	0·115
NH_3-N in aqueous extract	—	0·033	0·038	0·038	0·050

of $CaCO_3$ the reaction proceeds a stage further than in Exp. 1, both as judged by the amount of acids and gases formed as well as by the percentage losses of the straw constituents. Larger amounts of cellulose (20–23 %) and xylan (25–26 %) have been decomposed, and the acids formed amount to about 9·0 g. of which about 6·1 g. are acetic and the rest butyric.

Table II. *Decomposition of rice straw in presence of $CaCO_3$ (no nitrogen added).*

	100 g. original straw	After			
		1 month	2 months	3 months	4 months
	g.	g.	g.	g.	g.
		(A) Losses of constituents.			
Dry matter less ash	81·15	13·45	14·25	15·64	16·25
Straw cellulose (including xylan)	45·45	7·95	8·25	8·88	9·65
Xylan in cellulose	11·97	1·65	1·68	1·70	1·72
Xylan in polyuronides	9·50	2·40	2·84	3·46	3·93
Total xylans	21·47	4·05	4·52	5·16	5·65
Lignin (direct)	10·89	0·27	0·46	0·72	0·81
Lignin (after hydrolysis)	9·31	0·71	0·81	0·85	0·87
Protein	2·94	0·61	0·65	0·67	0·68
Gases (volumes in ml.):		(B) Products formed.			
CO_2	—	785	820	890	990
CH_4	—	40	50	55	65
Acetic acid	—	5·68	5·81	5·92	6·12
Butyric acid	—	2·45	2·50	2·62	2·83
p_H of medium	7·0	5·0	4·9	4·8	4·8
		(C) Nitrogen distribution.			
N content of original straw	0·469	—	—	—	—
Protein-N in residue	—	0·373	0·380	0·370	0·361
Protein-N in aqueous extract	—	0·070	0·065	0·070	0·080
NH_3-N in aqueous extract	—	0·020	0·025	0·025	0·025

It will be noted that even in presence of $CaCO_3$ the p_H of the medium falls below 5·0. This is in agreement with the p_H of bicarbonate mixtures in presence of CO_2, especially under pressures higher than atmospheric [Banta and Pomeroy, 1934]. As a result of the fall in p_H, further decomposition and gas production have been arrested, and only 1055 ml. of gas, mostly CO_2, have been evolved. The proportions of protein-nitrogen going into solution (16–18 %) and of ammoniacal nitrogen (about 5 %) are lower in presence of $CaCO_3$ than without it.

Exp. 3. Decomposition of rice straw in presence of $CaCO_3$ and ammonium carbonate. 5 g. $CaCO_3$ and ammonium carbonate to the extent of 1 % nitrogen on the straw taken were added to 20 g. portions of rice straw. The other experimental details were the same as in the previous experiments. The results are shown in Table III.

Table III. *Decomposition of rice straw in presence of $CaCO_3$ and ammonium carbonate (1 °/o N).*

	100 g. original straw g.	After				
		1 month g.	2 months g.	3 months g.	4 months g.	6 months g.
			(A) Losses of constituents.			
Dry matter less ash	81·15	14·45	16·90	20·15	24·20	28·50
Straw cellulose (including xylan)	45·45	5·90	9·67	15·06	17·77	20·60
Xylan in cellulose	11·97	1·22	2·26	3·85	4·05	4·86
Xylan in polyuronides	9·50	3·67	4·79	5·22	6·11	6·56
Total xylans	21·47	4·89	7·05	9·07	10·16	11·42
Lignin (direct)	10·89	0·95	1·21	1·41	1·52	1·61
Lignin (after hydrolysis)	9·31	1·02	1·10	1·26	1·33	1·35
			(B) Products formed.			
Gases (volumes in ml.):						
Total CO_2 evolved	—	685	1510	3240	3525	3735
Correction for CO_2 evolved from carbonates	—	630	1330	2955	3060	3225
Net CO_2 evolved	—	55	180	285	465	510
CH_4 evolved	—	35	165	245	430	475
H_2 evolved	—	5	15	20	25	30
Total volume of gases	—	95	360	550	920	1015
Gases (weights in g.):						
CO_2	—	0·11	0·36	0·57	0·92	1·01
CH_4	—	0·03	0·12	0·18	0·31	0·34
Acetic acid	—	8·88	10·43	11·03	12·83	14·23
Butyric acid	—	1·45	1·76	3·30	4·33	5·97
Total weight of products	—	10·47	12·67	15·08	18·39	21·55
p_H of medium	7·8	5·4	5·2	5·0	5·0	4·8

They show that a mixture of $CaCO_3$ and ammonium carbonate at 1 % N on straw is satisfactory from the point of view of losses of the different constituents, about 45–50 % of cellulose, 45–47 % of the total xylan and 14–15 % of the lignin being decomposed, but fails to keep the medium from turning acid, in spite of excess of $CaCO_3$, being present at the end of the reaction. This result is probably due to insufficiency of the ammonium carbonate added to neutralise the acids formed and the inability of $CaCO_3$, as already noted under Exp. 2, to prevent the fall in p_H. The p_H of the medium falls to 4·8 and the acids accumulate to 20·2 g. of which 14·23 g. are acetic and the remainder butyric. 3735 ml. of CO_2 and 475 ml. of CH_4 are evolved, most of the CO_2 coming from the interaction of the organic acids formed with the added $CaCO_3$. By comparison with the results of Exps. 4 and 6, the small amount of CH_4 evidently results from the fall in p_H and consequent inhibition of the decomposition of organic acids formed into gaseous products.

Exp. 4. Decomposition of rice straw in presence of ammonium carbonate: $CaCO_3$ absent. To 20 g. portions of rice straw was added ammonium carbonate at the rate of 2 % nitrogen on the straw taken, but no $CaCO_3$. Other experimental details were as before. The initial p_H was 7·8–7·9. In a few cases, the progress of the reaction was hindered by too rapid an accumulation of acids in the early stages and a corresponding decrease in the rate of gas production; but in others the two reactions proceeded at comparable rates and the p_H was maintained at about 7·4–7·8. This improved considerably the yield of combustible gases.

The results of the experiment are given in Table IVA. A comparison of these figures with those obtained in Exp. 3 using $CaCO_3$ and ammonium carbonate at

Table IVA. *Decomposition of rice straw in presence of ammonium carbonate (no $CaCO_3$ added).*

	100 g. original straw g.	After				
		1 month g.	2 months g.	3 months g.	4 months g.	6 months g.
			(A) Losses of constituents.			
Dry matter less ash	81·15	15·15	17·55	22·95	26·65	35·20
Straw cellulose (including xylan)	45·45	4·38	6·63	10·47	13·67	21·26
Xylan in cellulose	11·97	1·54	2·35	3·25	3·73	6·36
Xylan in polyuronides	9·50	4·10	4·72	5·53	6·23	6·43
Total xylans	21·47	5·64	7·07	8·78	9·96	12·79
Lignin (direct)	10·89	0·87	1·15	1·47	2·02	3·04
Lignin (after hydrolysis)	9·31	1·20	1·43	1·99	2·37	2·60
Protein	2·94	0·87	0·86	0·87	0·87	0·85
			(B) Products formed.			
Gases (volumes in ml.):						
Total CO_2 evolved	—	2705	3390	6220	7,320	10,290
Correction for CO_2 from carbonates by action of acids present	—	2700	3040	2430	2,225	460
Net amount of CO_2 evolved	—	5	350	3790	5,095	9,830
CH_4 evolved	—	20	360	3950	4,855	9,585
H_2 evolved	—	—	5	25	50	90
Total volume of gases	—	25	715	7765	10,000	19,505
Gases (weights in g.):						
CO_2	—	0·02	0·69	7·49	10·07	19·44
CH_4	—	0·02	0·26	2·83	3·48	6·87
Acetic acid	—	6·65	6·43	4·90	4·51	1·07
Butyric acid	—	0·85	2·52	2·36	2·13	0·23
Total weight of products	—	7·54	9·90	17·58	20·19	27·61
p_H of medium	7·9	6·6	6·4	7·0	7·2	7·8
			(C) Nitrogen relationships.			
N in original straw	0·469	—	—	—	—	—
Protein-N in residue	—	0·329	0·322	0·333	0·348	0·333
Protein-N in aqueous extract	—	0·153	0·138	0·138	0·188	0·237
Total protein-N after fermentation	—	0·482	0·460	0·471	0·536	0·570
N immobilised per 100 g. straw or nitrogen factor	—	0·013	nil	0·002	0·067	0·101
Nitrogen equivalent	—	0·086	nil	0·009	0·219	0·252

1 % nitrogen on the straw shows that the essential difference lies in the degree of gas production in the two cases. Whereas in Exp. 3 the acids formed accumulated to 20·2 g. and the p_H fell to 4·8, the total acids formed in the present experiment gave a maximum value of 8·95 g. at the end of 2 months and then decreased steadily to 1·3 g. at the end. Corresponding with this variation in the amount of acids, the p_H of the medium first showed a fall from 7·9 to 6·4 at the end of 2 months after which it registered a steady rise to 7·8. Evolution of appreciable amounts of combustible gases, principally methane, began at the end of the second month and showed a rapid increase in that period when the acids were rapidly falling.

The gas evolution, corrected for CO_2 evolved by the interaction of added carbonates with the organic acids formed, amounted in the present experiment to 19,505 ml. containing 9585 ml. methane. In Exp. 3 the corresponding figures were 1015 and 475. That the much larger amount of methane produced in the present experiment has in all probability resulted from the decomposition of the organic acids which accumulated in Exp. 3 is shown by a comparison of the total possible methane production in the two cases, given in Table IV B.

Table IV B.

	Exp. 3	Exp. 4
Acetic acid present	14·23 g.	1·07 g.
Butyric acid present	5·97 g.	0·23 g.
CH_4 evolved	475 ml.	9585 ml.
CH_4 equivalent to acetic acid present	5311 ml.	399 ml.
CH_4 equivalent to butyric acid present	4588 ml.	176 ml.
Total capacity for methane production	10374 ml.	10,160 ml.

The similarity of the total volumes of methane derivable from the organic acids produced in the two experiments supports the hypothesis that the lower organic acids are the chief intermediate compounds formed in the breakdown of the constituents of straw, and that the organic acids are further split up into CH_4 and CO_2 [*cf.* Buswell and Boruff, 1932; Tarvin and Buswell, 1934]. The evidence obtained in these experiments does not support the theory that CO_2 and H_2 are the first products of decomposition and that a portion of the CO_2 is reduced by hydrogen to CH_4 and organic acids.

It is worth noting that if the total yield of CO_2 in both Exps. 3 and 4 is corrected for that portion evolved from added carbonates by the action of acids formed, the CO_2/CH_4 ratio is almost unity at successive stages of decomposition. Such a constant ratio may not be expected on the basis of a partial reduction of CO_2 by H_2 but agrees well with the decomposition of organic acids into equivalent amounts of CH_4 and CO_2.

The total yield of gases is about 19,500 ml. per 100 g. of straw, and allowing for the residual 1·3 g. of acid present, the yield will rise to about 20,500 ml., containing about 50 % methane. This corresponds to a yield of about 7200 cb. ft. of gas per ton of straw.

Another interesting point to be noted from Table IV is the low nitrogen requirement for anaerobic digestion. Rege [1927] and Richards and Norman [1931] found that for aerobic decomposition the "nitrogen factor" (the amount of nitrogen converted from the mineral into protein form per 100 g. of material taken) ranged from 0·7 to 0·9 for rice straw, whereas the highest factor obtained in the present experiment was about 0·1. The above authors found a nitrogen equivalent (the amount of nitrogen immobilised per 100 g. of material destroyed) of about 2 for rice straw. For anaerobic decomposition, the nitrogen equivalent varies from nil to 0·25.

Exp. 5. Decomposition of rice straw in presence of varying amounts of sodium nitrate. It is well known that under anaerobic conditions nitrates are rapidly denitrified in presence of organic matter, leaving an alkaline residue which serves the purpose of a neutralising agent. To 20 g. portions of rice straw was added sodium nitrate at the rate of 0·5, 1, 2 and 3 % nitrogen on the straw. No $CaCO_3$ was added and other experimental details were as before.

The data obtained are given in Table V. They show that by the use of sodium nitrate in proper amounts, successful decomposition of rice straw could be brought about which would compare favourably with the decomposition accomplished by the use of ammonium carbonate. The amount of decomposition increases with increasing doses of $NaNO_3$ up to an amount representing 2 % N on the straw, after which the reaction becomes presumably too alkaline to permit favourable decomposition.

Table V. *Decomposition of rice straw with different doses of sodium nitrate.*

	100 g. original straw g.	$NaNO_3$ at 0·5 % N g.	$NaNO_3$ at 1 % N g.	$NaNO_3$ at 2 % N g.	$NaNO_3$ at 3 % N g.
		(A) Losses of constituents.			
Dry matter less ash	81·15	15·02	20·42	28·26	18·34
Straw cellulose (including xylan)	45·45	7·02	11·83	12·90	8·38
Xylan in cellulose	11·97	2·81	4·05	4·41	2·98
Xylan in polyuronides	9·50	5·27	5·38	5·98	5·25
Total xylans	21·47	8·08	9·43	10·39	8·23
Lignin (direct)	10·89	2·18	2·29	2·39	2·31
Lignin (after hydrolysis)	9·31	1·90	2·02	2·41	2·57
Protein	2·94	0·86	0·94	1·10	1·12
		(B) Products formed.			
Gases (volumes in ml.):					
CO_2 evolved and in solution	—	1860	3310	6260	3885
CH_4	—	210	410	2630	150
H_2	—	40	65	105	30
N_2	—	225	440	920	280
Total volume of gases	—	2335	4225	9915	4345
Gases (weights in g.):					
CO_2	—	3·68	6·54	12·38	7·68
CH_4	—	0·15	0·29	1·89	0·11
H_2	—	—	—	0·01	—
N_2	—	0·29	0·53	1·24	0·54
Total weight of gases	—	4·12	7·36	15·52	8·33
Acetic acid	—	4·05	7·41	9·10	7·32
Butyric acid	—	0·54	1·70	1·76	0·84
Total weight of products	—	8·71	16·47	26·38	16·49
p_H of medium	—	5·0	6·2	7·4	8·6

The action of sodium nitrate is evidently two-fold, the first stage being denitrification of nitrate (accompanied by evolution of free nitrogen) and oxidation of organic matter (with evolution of CO_2) and the second being the usual type of anaerobic decomposition with formation of organic acids and evolution of combustible gases met with in the experiments above described. No methane or hydrogen could be detected till all the nitrate had been reduced and the evolution of free nitrogen had ceased. It is interesting to compare in this connection the observations of Elma *et al.* [1934] who found that the oxidation-reduction potential of a nitrate or nitrite medium undergoing denitrification by *Micrococcus denitrificans* was maintained at a steady level depending on the p_H of the medium till all the nitrite or nitrate had been reduced, after which it showed an immediate and great fall. It is suggested that this stage corresponds in the present experiment to the starting of the second stage of anaerobic decomposition accompanied by the formation of acids and evolution of combustible gases; but the point needs further examination.

It would appear from Table V that sodium nitrate at the rate of 2 % N on the straw would, after denitrification, yield enough alkaline residue to neutralise the acids formed in the second stage of decomposition and maintain the alkaline reaction so essential for the further progress of the reaction. With a view to examining the course of the decomposition in more detail, the experiments with sodium nitrate were repeated at the above concentration, samples being examined after definite time intervals.

Exp. 6. Decomposition of rice straw in presence of sodium nitrate equivalent to 2 °/₀ N on straw. A number of bottles were set up with 20 g. portions of rice straw and sodium nitrate at the rate of 2 % N on the straw, and samples were taken out for analysis at intervals. The results obtained are given in Table VI.

Table VI. *Decomposition of rice straw by $NaNO_3$ equivalent to 2 °/₀ N on straw.*

	100 g. original straw g.	After			
		1 month g.	2 months g.	4 months g.	6 months g.
(A) Losses of constituents.					
Dry matter less ash	81·15	15·15	19·65	30·25	31·95
Straw cellulose (including xylan)	45·45	6·13	9·58	15·60	16·66
Xylan in cellulose	11·97	1·81	2·27	4·47	4·72
Xylan in polyuronides	9·50	4·95	5·39	5·67	6·13
Total xylans	21·47	6·76	7·66	10·14	10·85
Lignin (direct)	10·89	1·74	1·97	2·31	2·89
Lignin (after hydrolysis)	9·31	1·84	1·89	1·92	2·15
Protein	2·94	1·08	1·08	1·18	1·09
(B) Products formed.					
Gases (volumes in ml.):					
CO_2 evolved and in solution	—	2575	2815	5925	8095
CH_4 evolved and in solution	—	—	45	2655	5850
H_2	-	—	25	50	90
N_2	—	710	700	815	720
Total volume of gases	—	3285	3585	9445	14,755
Gases (weights in g.):					
CO_2	—	5·09	5·57	11·71	16·01
CH_4	—	—	0·03	1·90	4·19
H_2	—	—	—	—	0·01
N_2	—	8·88	8·76	10·20	9·00
Total weight of gases	—	13·97	14·36	23·81	29·21
Acetic acid	—	2·79	6·00	5·36	3·64
Butyric acid	—	—	0·63	0·79	—
Total weight of products	—	16·76	20·99	29·96	32·85
p_H of medium	—	7·2	7·4	7·8	8·2
(C) Nitrogen distribution.					
N in original straw	0·469	—	—	—	—
N added as $NaNO_3$	2·000	—	—	—	—
Protein-N in residual straw	—	0·297	0·297	0·281	0·295
Protein-N in aqueous extract	—	0·163	0·150	0·175	0·175
NH_3-N in aqueous extract	—	0·050	0·025	0·038	0·025
Total protein-N and NH_4-N	—	0·510	0·472	0·494	0·495
N fixed from added nitrate	—	0·041	0·003	0·025	0·026

A comparison of the results obtained in Exps. 6 and 4, using sodium nitrate and ammonium carbonate respectively, shows essential differences in the course of the two reactions, especially in the earlier stages. With sodium nitrate denitrification of nitrate and oxidation of the organic matter proceed quite rapidly. At the end of one month the sodium nitrate culture has oxidised about 13 % of cellulose and 31 % of total xylans, as compared with 8·4 and 26 % respectively in the ammonium carbonate culture. However, with sodium nitrate only 2·8 g. of organic acid are present at the end of one month while 7·5 g. are present after the same period when ammonium carbonate is used. After the denitrification stage is completed the usual type of anaerobic degradation begins, as evidenced by the accumulation of organic acids and evolution of CH_4.

It is interesting to note from Table VI that practically the whole of the added nitrate is lost, so far as the non-gaseous portion is concerned, the actual amount of nitrate-nitrogen converted into protein or ammoniacal form being negligible. In spite of the comparatively large amount of protein in solution, the level of ammoniacal nitrogen is quite low, there being only 5–10 mg. in a volume of 200 ml. This low level of ammoniacal nitrogen, even in cultures where decomposition has been active, confirms what has already been noted, namely, that a small supply of available nitrogen is enough to sustain anaerobic decomposition and that there is no advantage gained by adding larger amounts of ammonium salts than may be used to neutralise the organic acids formed.

DISCUSSION.

The experiments outlined above show that the first phase of anaerobic decomposition is the rapid formation of organic acids and considerable loss of organic matter in the early stages. The evolution of combustible gases, *e.g.* methane, appears to be a secondary reaction, and from the relative amounts of acids and gases produced under differing conditions it seems that the organic acids first formed are decomposed further into CO_2 and CH_4, though at a rate much slower than the speed of acid formation. This second stage of gas production appears to be more sensitive to the influence of p_H than the first and proceeds best at a slightly alkaline reaction (about p_H 7·5). The rapid progress of anaerobic decomposition therefore depends primarily on the maintenance of a favourable p_H range, which is much narrower than that for aerobic decomposition.

A second point brought out by these experiments is the very low nitrogen requirement for anaerobic decomposition. Hutchinson and Richards [1921; 1925] have shown that for rapid conversion of waste materials into manure under aerobic conditions, the addition of nitrogen at the rate of about 0·7 % on the material taken is necessary. With anaerobic decomposition, however, additions of nitrogen above a low minimum value appear to exert no direct beneficial influence. Using rice straw, about 30 % of the total protein goes into solution even in the early stages and about 5–10 % is converted into ammonia. The insoluble residue at the end of the decomposition contains only about 70 % of the original nitrogen present in the straw, even though large additions of ammonia in the form of ammonium carbonate are made. This no doubt is [illegible]ble on the assumption that anaerobic decomposition is one of reduction to [illegible] products and hence very little synthesis of added ammonia into protein [illegible]ce under these conditions. The bacterial population is conceivably o[illegible]ture and there is probably a continuous cycle of microbial synthes[illegible]gration, making the nitrogen requirement much less than under aerob[illegible]s. It has been noted under Exp. 4 that even under the most favoura[illegible]ons, the nitrogen factor [Rege, 1927] is only of the order of 0·1, while the nitrogen equivalent [Richards and Norman, 1931] is about 0·2–0·25, as compared with the values of 0·7–0·9 and 1·8–2·0 respectively obtained for aerobic decomposition. In several cases the values have been negative, showing that under anaerobic conditions the net tendency is towards hydrolysis rather than towards synthesis of nitrogen compounds.

The apparent stimulating effect of additions of ammonium carbonate seen in Exps. 3 and 4 therefore seem to be due to its neutralising effect on the acids formed and the maintenance of an alkaline reaction rather than to the supply of available nitrogen.

The products obtained as a result of anaerobic decomposition have been of a simple character, the organic acids formed being mainly acetic and butyric and the gases evolved being mostly CO_2 and methane. Only negligible amounts of hydrogen are evolved at any stage. Where carbonates are used to neutralise the acids formed, nearly the whole of the gas first evolved is CO_2. Later, when the decomposition of organic acids into CO_2 and CH_4 occurs, larger and increasing proportions of CH_4 are formed, rising to 70–80 % of the gases evolved. When, however, allowance is made for the amount of CO_2 evolved from added carbonates and that retained in solution, the CH_4/CO_2 ratio is nearly unity at successive stages of the decomposition.

As regards the extent of decomposition, under optimum conditions 40–45 % of ash-free dry matter, about 45 % of cellulose and 60 % of the hemicelluloses, in terms of furfuraldehyde yield, are decomposed. The hemicelluloses are more rapidly attacked in the early stages, but at the end of the reaction there is a larger total amount of cellulose decomposed than of hemicelluloses. In this respect the course of decomposition is similar to that under aerobic conditions, though the relative percentages are lower [*cf.* Rege, 1927; Tenney and Waksman, 1929]. Under conditions favourable to the accumulation of acids it is possible to obtain as much as 20 g. of acids per 100 g. of straw taken. Under other conditions which favour gas production, over 20 litres of gas containing about 50 % methane could be obtained from the same amount of straw. This corresponds to a yield of about 7200 cb. ft. gas per ton of straw and is comparable with the yields of 4400 cb. ft. per ton of wheat straw and 9360 cb. ft. per ton of Nile sudd obtained by Richards and Amoore [1920].

A significant point brought out by these experiments is the degree to which lignin appears to have been decomposed. About 25–30 % of the lignin, as determined both directly on the material as well as after a preliminary hydrolysis with 5 % H_2SO_4 [Norman and Jenkins, 1933], has undergone decomposition. This is in marked contrast to the difficulty with which lignin is attacked under aerobic conditions. Boruff and Buswell [1934] have also found a similar considerable loss of lignin content, in some cases amounting to 50 % of the total lignin, when corn stalks were decomposed under anaerobic conditions. It should be noted however that much confusion exists at present in methods adopted for lignin determination and errors and differences of a serious nature may be obtained, due to differences in technique or the presence of interfering substances [Phillips, 1934; Norman and Jenkins, 1934].

SUMMARY.

1. The course of anaerobic decomposition of rice straw has been followed with reference to losses of its major constituents, such as cellulose, furfuraldehyde-yielding compounds, lignin, proteins *etc.*, and the formation of organic acids and gases.

2. Of the straw constituents about 60 % of the hemicelluloses, 45 % of the cellulose and 25–30 % of lignin are decomposed. The ultimate loss of ash-free dry matter is about 40–45 %.

3. Acetic and butyric acids, CO_2 and methane are the main products formed. From 100 g. of straw, either about 20 g. of organic acids or over 20 litres of gases containing about 50 % methane are obtainable. This is equivalent to about 7200 cb. ft. of gas yield per ton of straw.

4. The decomposition appears to proceed in two distinct stages, the first involving the formation of organic acids and the second the conversion of these into gaseous products. A slightly alkaline reaction, a p_H of about 7·5, is important for the progress of the second stage of decomposition. The uses of $CaCO_3$, $NaNO_3$ and ammonium carbonate for this purpose have been compared. Ammonium carbonate in sufficient amount to neutralise the acids formed was found most suitable.

5. The reaction with $NaNO_3$ proceeds in two stages, the first being denitrification of the nitrate and oxidation of the organic matter; the alkaline residue left after denitrification serves to neutralise the organic acids formed in the second stage, which is the usual type of anaerobic fermentation accompanied by the evolution of combustible gases.

6. While the supply of available nitrogen is of paramount importance for the aerobic decomposition of plant substances, it is relatively insignificant in anaerobic decomposition. 100 g. of rice straw require about 0·7 part of nitrogen for decomposition aerobically but only 0·1 part or even less under anaerobic conditions.

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REFERENCES.

Adeney (1932). *J. Roy. San. Inst.* **53**, 171.
Assoc. of Off. Agric. Chemists, U.S.A. (1930). Official and tentative methods of analysis (Washington).
Banta and Pomeroy (1934). *Sewage Works J.* **6**, 234.
Bartholomew (1929). *Soil. Sci.* **28**, 85.
Boruff and Buswell (1929). *Ind. Eng. Chem.* **21**, 1181.
—— —— (1934). *J. Amer. Chem. Soc.* **56**, 886.
Brown (1923). *J. Bact.* **8**, 245.
Buswell and Boruff (1932). *Sewage Works J.* **4**, 454.
Daikuhara and Imaseki (1907). *Bull. Imp. Agric. Expt. Stn., Japan*, **1**, No. 2.
Elma, Kluyver and Dalfsen (1934). *Biochem. Z.* **270**, 317.
Fowler and Joshie (1920). *J. Indian Inst. Sci.* **3**, 39.
Friedmann and Graeser (1933). *J. Biol. Chem.* **100**, 291.
Harrison and Iyer (1913). *Mem. Dept. Agric. India, Chemical Series*, **3**, 65.
—— —— (1914). *Mem. Dept. Agric. India, Chemical Series*, **4**, 1.
—— —— (1915). *Mem. Dept. Agric. India, Chemical Series*, **4**, 135.
—— —— (1916). *Mem. Dept. Agric. India, Chemical Series*, **5**, 1.
—— —— (1919). *Mem. Dept. Agric. India, Chemical Series*, **5**, 173.
—— —— (1920). *Mem. Dept. Agric. India, Chemical Series*, **5**, 181.
Heukelekian (1933). *Ind. Eng. Chem.* **25**, 1162.
Hutchinson and Richards (1921). *Min. Agric. London*, **28**, 398.
—— —— (1925). *Trop. Agric. (Ceylon)*, **64**, 24.

Itano and Arakawa (1927). *Ber. Ohara Inst. landw. Forsch.* (*Japan*), **3**, 331.
—— —— (1932). *Proc. Internat. Soc. Soil Sci.* **7**, 252.
Joachim and Kandiah (1929). *Trop. Agric.* (*Ceylon*), **72**, 253.
Langwell and Lymn (1923). *J. Soc. Chem. Ind.* **42**, 280.
—— —— (1932). *J. Soc. Chem. Ind.* **51**, 988.
Larson, Boruff and Buswell (1934). *Sewage Works J.* **6**, 24.
Metzger (1929). *Soil Sci.* **27**, 305.
—— and Janssen (1928). *J. Amer. Soc. Agron.* **20**, 459.
Misu and Shimohira (1929). *Ann. Agric. Exp. Sta. Gov.-Gen. Chosen.* **4**, 65.
Nagaoka (1905). *Bull. Coll. Agric. Tokyo*, **6**, 285.
Norman (1929). *Biochem. J.* **23**, 1353.
—— and Jenkins (1933). *Biochem. J.* **27**, 818.
—— —— (1934). *Biochem. J.* **28**, 2147, 2160.
Osugi, Yoshie and Komatsubara (1931), *Mem. Coll. Agric. Kyoto Imp. Univ.* **12**, 1.
—— —— —— (1932). *Proc. 2nd Internat. Congr. Soil Sci.* **3**, 107.
Panganiban (1923). *Phil. Agric.* **12**, No. 2.
Phillips (1934). *J. Assoc. Off. Agric. Chem.* **17**, 277.
Rege (1927). *Ann. Appl. Biol.* **14**, 1.
Richards and Amoore (1920). Unpublished data.
—— and Norman (1931). *Biochem. J.* **25**, 1769.
Robinson (1930). *Soil Sci.* **30**, 197.
Rudolfs (1932). *Sewage Works J.* **4**, 444.
—— and Heukelekian (1932). *Ind. Eng. Chem.* **24**, 1312.
—— and Setter (1932). *Annual Rept. Dept. Water Supplies and Sewage Disposal, Bull.* No. 529 *of N.J. Agric. Exp. Sta., New Brunswick, N.J.*
Subrahmanyan (1927). *J. Agric. Sci.* **17**, 429, 449.
—— (1929). *J. Agric. Sci.* **19**, 627.
—— and Sreenivasan (1934). *Current Sci.* **2**, 432.
Symons and Buswell (1933). *J. Amer. Chem. Soc.* **55**, 2028.
Tarvin and Buswell (1934). *J. Amer. Chem. Soc.* **56**, 1751.
Tenney and Waksman (1929). *Soil Sci.* **28**, 55.
—— —— (1930). *Soil Sci.* **30**, 143.
Virtanen and Pulkki (1928). *J. Amer. Chem. Soc.* **50**, 3138.
Whitehead and O'Shaughnessy (1931). *Proc. Inst. Civil Eng.* **233**, 38.

LXII. THE SYNTHESIS OF AMINO-ACIDS.

I. PIPERIDINE AND DIETHYLAMINE AS CATALYSTS IN THE CONDENSATION OF AROMATIC ALDEHYDES WITH HYDANTOINS.

By WILLIAM JOHN BOYD and WILLIAM ROBSON.

From the Department of Physiology, University of London King's College.

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An important method of synthesising aromatic α-amino-acids is that of Wheeler and Hoffman [1911] which involves in its first stage the condensation of the appropriate aldehyde with hydantoin, the reaction being carried out in hot glacial acetic acid containing anhydrous sodium acetate. The yield of the condensation product so obtained varies considerably according to the aldehyde used. For example benzalhydantoin may be prepared in a yield of 80 % of the theoretical whilst indolalhydantoin is only obtainable in a yield of 47 %. Majima and Kotake [1922], who first prepared the latter compound from indole-3-aldehyde and hydantoin, found that it was necessary to substitute acetic anhydride for acetic acid in the condensation. Besides indolalhydantoin they also obtained unspecified amounts of acetylated indolalhydantoin, unchanged indole-3-aldehyde and another unidentified substance. From a study of the same reaction in this laboratory it appears, however, that the low yield obtained is due not so much to incomplete reaction or to acetylation as to the formation of tarry products of complex character.

Whatever be the cause, such a loss of material becomes a matter of no small importance when the parent aldehyde, *e.g.* indole-3-aldehyde, is not easy of access. Since moreover a scheme of study in this laboratory involved the condensation of such aldehydes with hydantoin, it was decided, as a first measure, to investigate the possibility of carrying out the reaction in solvents other than glacial acetic acid and with catalysts other than anhydrous sodium acetate.

The reaction under study falls into the class of condensations between an aldehydic and a reactive methylene group with elimination of a molecule of water. From a consideration of the reaction in its various aspects there seemed to be no apparent reason why the organic bases, diethylamine, aniline, piperidine, nicotine *etc.*, used with such success by Knoevenagel [1894] for effecting similar condensations, should not be equally applicable in this case.

That these bases can be so used has now been proved experimentally. It soon became clear however that in specific cases individual catalysts are more effective than others. For example, in attempting the condensation of benzaldehyde, *p*-hydroxybenzaldehyde or *p*-methoxybenzaldehyde with hydantoin by means of piperidine, using pyridine as solvent, poor yields accompanied by much tar were obtained. On substituting 3-acetyl-2-thiohydantoin for ordinary hydantoin, there resulted practically quantitative yields of condensation products of a high degree of purity. Replacement of piperidine by diethylamine as the catalyst employed gave good yields in the case of ordinary hydantoin, and it is notable that the recrystallised product was not straw-coloured, as when made by the Wheeler-Hoffman technique, but colourless. A series of experiments on the above lines with 1-phenylhydantoin gave results analogous to those obtained with ordinary hydantoin; owing to its high cost however 1-phenylhydantoin is of little importance in schemes of synthesis of α-amino-acids. The substitution of absolute alcohol for pyridine as the solvent, in conjunction with diethylamine as catalyst, gave only a 25 % yield of benzalhydantoin after heating for 24 hours; it is probable that a longer period of heating would improve the yield.

The results described in the experimental section may be briefly summarised thus:

Reactants	% yield of condensation product	
	Piperidine	Diethylamine
Benzaldehyde and hydantoin	33	76
,, acetylthiohydantoin	92·5	
Anisaldehyde and hydantoin	14	94
,, acetylthiohydantoin	95	
p-Hydroxybenzaldehyde and hydantoin	25	65
,, acetylthiohydantoin	95–100	

It will be clear that the work herein described is capable of extension in several directions. For example, whilst it will be noted that the condensations described have all been carried out at 100°, nevertheless evidence is not lacking that they may proceed at a very much lower temperature. Moreover, of the bases originally employed by Knoevenagel only diethylamine and piperidine have been used up to the present, and it is proposed to extend the study to include others of those mentioned above. Finally, reference may be made to one other important phase of this problem. Whilst the Wheeler-Hoffman process has been of considerable value in the synthesis of aromatic α-amino-acids the applicability of the method to the synthesis of aliphatic α-amino-acids has never been demonstrated. The introduction of new solvents and catalysts for promoting the reaction between aldehydes and hydantoin suggests new lines of attacking the problem of synthesising aliphatic α-amino-acids by way of the hydantoins. This aspect of the investigation is receiving attention.

EXPERIMENTAL.

Experiments involving piperidine as catalyst.

4-Benzalhydantoin. Pure benzaldehyde (2·2 g.), hydantoin (2·0 g.) and piperidine (2 ml.) were boiled together for 1 hour. The product was treated with 100 ml. of hot water and filtered, whereby some tarry material was removed. The filtrate was neutralised with dilute hydrochloric acid, whereupon benzalhydantoin separated in characteristic rhombic plates. It was recrystallised from dilute alcohol and had M.P. 220°; N 14·72 %. Wheeler and Hoffman [1911] give the same melting-point. $C_{10}H_8O_2N_2$ requires 14·90 % N. The yield was 1·6 g. or 33 % of the theoretical value.

Benzalthiohydantoin. The acetylthiohydantoin required in this experiment was prepared from glycine and ammonium thiocyanate as described by Johnson and Nicolet [1911]. Benzaldehyde (2·5 g.) and acetylthiohydantoin (3 g.) were dissolved in pyridine (5 ml.). On adding piperidine (3 ml.), the mixture became warm and deep yellow in colour. It was boiled for 2 minutes and then water (100 ml.) was added. The resulting aqueous solution was acidified by the addition of acetic acid and warmed until the oily mass which separated had given place to a yellow crystalline deposit. The mixture was cooled and kept overnight. The yellow crystals which had then separated were collected, washed with cold water and recrystallised from dilute alcohol. They weighed only 2·4 g., but further crops were obtained after the mother-liquor had stood for some days. The total yield was 3·65 g. or about 92·5 % of the theoretical figure; M.P. 258° (Johnson and Nicolet give 258°). Found N 13·59 %. $C_{10}H_8ON_2S$ requires N 13·73 %.

Benzalhydantoin from the above benzalthiohydantoin. A portion of the benzalthiohydantoin (1·3 g.), synthesised according to the procedure detailed above, was boiled with 50 % chloroacetic acid (20 ml.) for 3 hours. The resulting clear solution was evaporated to small volume *in vacuo* and the residue diluted with water. A slightly yellow crystalline substance separated and after some time was filtered off and washed with cold water. Without recrystallisation, it weighed 0·92 g., melted at 220° and contained 14·8 % N. The yield, referred back to the original acetylhydantoin, was 77 % of the theoretical.

p-*Methoxybenzalhydantoin.* This experiment was carried out as described for benzalhydantoin. From 2·64 g. of anisaldehyde, 0·45 g. (14 %) of *p*-methoxybenzalhydantoin was obtained together with much tar. After recrystallisation from dilute alcohol the M.P. was 243° (Wheeler and Hoffman quote 243–244°). Found N 12·79 %. $C_{11}H_{10}O_3N_2$ requires N 12·85 %.

p-*Methoxybenzalthiohydantoin.* *p*-Methoxybenzalthiohydantoin was prepared from anisaldehyde (2·5 g.) and acetylthiohydantoin (3 g.) as described for benzalthiohydantoin. The yield of uncrystallised product was 4·33 g. or 95 % of the theoretical calculated on the acetylthiohydantoin taken. After crystallisation from alcohol, the product melted at 256° (Johnson and O'Brien [1912] give 257°). Found N 12·14 %. $C_{11}H_{10}O_2N_2S$ requires N 11·97 %.

p-*Methoxybenzalhydantoin from the above* p-*methoxybenzalthiohydantoin.* *p*-Methoxybenzalthiohydantoin (5 g.), obtained as in the preceding experiment but without recrystallisation, was boiled with a mixture of 50 % chloroacetic acid (50 ml.) and acetic acid (150 ml.) for 4 hours. The substance was very insoluble and only went into solution gradually. The resulting solution was evaporated to small volume *in vacuo* and the residual fluid was diluted with water. A yellow crystalline substance separated from the cold solution and was, after some time, collected and washed with cold water. The yield was 94 %. It melted at 247° before recrystallisation, and contained 12·79 % N. Referred back to the acetylthiohydantoin used for the preparation of the *p*-methoxybenzalthiohydantoin, the yield was equivalent to 85–90 %.

p-*Hydroxybenzalhydantoin.* This substance was prepared in the same manner as benzalhydantoin. From 2·4 g. of *p*-hydroxybenzaldehyde, 0·75 g. (25 %) of the desired product was obtained. The product separated from alcohol in needles which melted at 315°. Found N 13·62 %. $C_{10}H_8O_3N_2$ requires N 13·72 %.

p-*Hydroxybenzalthiohydantoin.* The compound was prepared from *p*-hydroxybenzaldehyde (2·5 g.) and acetylthiohydantoin (3 g.) as described for benzalthiohydantoin. The yield of recrystallised product was 4·2 g. which is practically theoretical. The purified product melted at 305° and contained 12·82 % N. $C_{10}H_8O_2N_2S$ requires N 12·73 %.

p-*Hydroxybenzalhydantoin from* p-*hydroxybenzalthiohydantoin.* *p*-Hydroxybenzalthiohydantoin (4·0 g.), obtained in the preceding experiment but not recrystallised, was boiled with 50 % chloroacetic acid (50 ml.) for 3 hours. The solution was evaporated to small bulk *in vacuo* and the residual liquor diluted with water. A yellow crystalline substance separated; this was collected after some time and washed with cold water. After crystallisation from alcohol it weighed 2·9 g., melted at 314° and contained 13·84 % N. The yield of the desulphurised hydantoin was thus 85 % of the theoretical calculated on the weight of the acetylthiohydantoin used.

Experiments involving diethylamine as catalyst.

Benzalhydantoin. Benzaldehyde (5·5 g.), hydantoin (4·5 g.), pyridine (5 ml.) and diethylamine (6 drops) were heated together at 100° for 22 hours in a closed bottle. On cooling the mixture became solid. The contents of the bottle were dissolved in alcohol and evaporated to dryness. The product so obtained was crystallised from alcohol and it then melted at 220° and contained 14·95 % N. The yield of recrystallised material was 6·4 g. or 76 % of theory.

Another experiment, carried out under the same conditions but with the addition of 0·5 ml. of water, gave only 4·0 g. of product.

A third experiment carried out under the same conditions as the first, the temperature being raised to 120°, yielded a slightly sticky product which gave 70 % of the theoretical amount of recrystallised material.

p-*Methoxybenzalhydantoin.* Anisaldehyde (6·0 g.), hydantoin (4·5 g.), pyridine (5 ml.) and diethylamine (3 ml.) were heated together at 100° for 22 hours in a closed bottle. The product was worked up as in the preparation of benzalhydantoin by means of diethylamine. The *p*-methoxybenzalhydantoin was not recrystallised but was washed with a little cold benzene and dried. It then melted at 240–244° and contained 12·82 % N. The yield was 94 % of the theoretical calculated on the hydantoin used.

p-*Hydroxybenzalhydantoin.* A mixture of *p*-hydroxybenzaldehyde (6·0 g.), hydantoin (4·5 g.), pyridine (5 ml.) and diethylamine (3 ml.) was heated in a closed bottle at 100° for 22 hours. The product was worked up as in the analogous preparation of benzalhydantoin. It was recrystallised from alcohol after being washed with cold benzene. In this way 5·1 g. of *p*-hydroxybenzalhydantoin were obtained. It melted at 312°. The yield approximated to 65 % of the theoretical.

SUMMARY.

The original method of Wheeler and Hoffman of effecting the condensation of aromatic aldehydes with hydantoins by heating a mixture of these substances in glacial acetic acid containing anhydrous sodium acetate may be modified with advantage.

Instead of the mixture of acetic acid and sodium acetate, mixtures of pyridine and piperidine or of pyridine and diethylamine or even piperidine alone in some cases can be successfully substituted.

A table summarising the results is given. From this, it will be seen that the relative efficiencies of piperidine and diethylamine as catalysts vary according to the reactants involved.

REFERENCES.

Johnson and O'Brien (1912). *J. Biol. Chem.* **12**, 205.
—— and Nicolet (1911). *J. Amer. Chem. Soc.* **33**, 1972.
Knoevenagel (1894). *Liebig's Ann.* **281**, 25.
Majima and Kotake (1922). *Ber. deutsch. chem. Ges.* **55**, 3862.
Wheeler and Hoffman (1911). *Amer. Chem. J.* **45**, 368.

LXIII. THE SYNTHESIS OF AMINO-ACIDS.

II. SULPHIDES AS REDUCING AND HYDROLYTIC AGENTS IN THE HYDANTOIN SYNTHESIS OF AMINO-ACIDS.

By WILLIAM JOHN BOYD and WILLIAM ROBSON.

From the Department of Physiology, University of London King's College.

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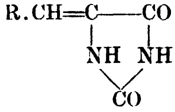
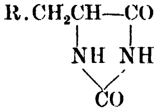
In the preceding paper of this series [1935] the authors have shown how the first, *i.e.* the condensation, stage of the Wheeler-Hoffman [1911] process of synthesising aromatic α-amino-acids may be modified, not only with regard to the catalyst but also the solvent employed, so that the yield and, in certain cases, the purity of the product may be improved.

The second and third stages of the synthesis consist respectively of the reduction of the unsaturated hydantoin (I), formed in the condensation stage referred to above, and of the hydrolysis of the resulting saturated hydantoin (II)



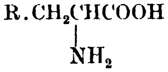
$$\begin{array}{l} R.CH{=}\underset{\displaystyle NH}{\underset{|}{C}}{-\!-}\underset{\displaystyle NH}{\underset{|}{CO}} \\ \qquad\quad \diagdown\!\diagup \\ \qquad\quad\; CO \end{array}$$

(I)



$$\begin{array}{l} R.CH_2\underset{\displaystyle NH}{\underset{|}{C}}H{-}\underset{\displaystyle NH}{\underset{|}{CO}} \\ \qquad\quad \diagdown\!\diagup \\ \qquad\quad\; CO \end{array}$$

(II)



$$R.CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$$

(III)

to the amino-acid (III). These two stages of reduction and hydrolysis, as shown by Wheeler and Hoffman, can be effected by one and the same reagent, *viz.* hydriodic acid and red phosphorus. By such means they obtained phenylalanine from benzalhydantoin and tyrosine from *p*-methoxybenzalhydantoin, whilst, more recently, Harington [1926] following the same procedure, prepared thyronine from 4-(4′-methoxyphenoxy)benzalhydantoin. When, however, the former workers attempted, by shortening the duration of treatment with hydriodic acid, to limit the process to one of reduction only, they found that, whilst the main product was the saturated hydantoin, it was invariably accompanied by amino-acid; in other words a certain degree of hydrolysis was inevitable. This difficulty can be readily overcome by the method adopted by Lamb and Robson [1931] who used a strictly limited quantity of hydriodic acid (dissolved in glacial acetic acid) and red phosphorus for the reduction of α-benzamidocinnamic acids to the corresponding α-benzamidophenylpropionic acids.

The method as described by Wheeler and Hoffman gives products in good yield and of excellent purity and, apart from the high cost of the hydriodic acid employed, has only one drawback as a general method. In the synthesis of certain aromatic α-amino-acids, *e.g.* tryptophan, the use of acid reducing agents such as hydriodic acid must be avoided. In such circumstances recourse is had to sodium amalgam. But the use of this reagent, in turn, has its disadvantages. Firstly, a high yield of the reduction product is only obtainable by the use of a very large excess of amalgam. Secondly, some hydrolysis always occurs during the process and the recovery of the resulting amino-acid from the solution, which contains a large quantity of sodium salts, is exceedingly difficult, if not impossible.

It was such practical difficulties as these, encountered during attempts to synthesise derivatives of tryptophan, which led us to investigate whether it was possible to carry out the reduction stage of the Wheeler-Hoffman synthesis by reagents other than those named above.

Amongst the many possibilities which suggested themselves to us was ammonium sulphide. In this connection we had, so far as we knew, no precedent for supposing that hydrogen sulphide would reduce a compound of type I to one of type II, but we were encouraged to attempt the experiment by the observation of Harries and Weiss [1903] that ordinary hydantoin can be hydrolysed to glycine by heating it with ammonium sulphide at 150° for 2 hours. On heating benzalhydantoin in a closed vessel with 16 % ammonium sulphide solution for 72 hours at 100° we obtained a mixture consisting of phenylalanine (50–60 % of theory) and benzylhydantoin (30–40 % of theory). A similar yield of amino-acid was obtained after only 3 hours by carrying out the reaction at 180°. Under similar conditions, benzalthiohydantoin gave partly α-thiocarbamido-β-phenylpropionic acid and partly the corresponding thiohydantoin, the former of which on being boiled with hydrochloric acid is reconverted into the thiohydantoin, whilst the latter compound, as shown by Johnson and Nicolet [1913], can be desulphurised to give benzylhydantoin by treatment with boiling chloroacetic acid.

From such results it was clear that we had found an excellent reducing agent, the use of which would eliminate many of the difficulties attached to the use of hydriodic acid and sodium amalgam. Since however the action of aqueous ammonium sulphide on the unsaturated hydantoin yields a mixture of products, it appeared essential to determine the conditions whereby maximum yields of the reduced hydantoin and of the amino-acid could be obtained.

The problem of obtaining a maximum yield of the reduced hydantoin was solved in the following way. Since certain unsaturated hydantoins were found to be extremely insoluble in aqueous ammonium sulphide even at 100°, an attempt was made to overcome this difficulty by carrying out the reaction at a higher temperature and by adding to the reaction mixture alcohol, pyridine or some such solvent miscible with water. Nevertheless, owing to the solubility factor, the amount of unsaturated hydantoin which could be treated by a given volume of ammonium sulphide solution was much less than the equivalent of the reducing power of the solution. In order therefore to make the method more convenient for the reduction of larger quantities of the more insoluble unsaturated hydantoins the possibility of effecting their reduction by hydrogen sulphide in anhydrous solvents, *e.g.* ethyl alcohol, pyridine *etc.*, was explored. Briefly it may be stated that the maximum yields of reduced hydantoin were obtained by heating the unsaturated hydantoin

(1) at 100° for 48–72 hours in pyridine saturated with hydrogen sulphide at 0°;

(2) at 58° in a mixture of aqueous ammonium sulphide and alcohol for a longer period;

(3) at 100° for 30–35 hours in an alcoholic solution of sodium or barium sulphide, the solution being saturated with hydrogen sulphide at 0° at the beginning of the experiment and again at that temperature after 16 hours.

The yields obtained by such methods was approximately the theoretical.

The optimum conditions for effecting the reduction of the unsaturated hydantoin having been determined, attention was next directed to the problem of converting the reduced hydantoin (II) into the amino-acid (III).

Reduced hydantoins can be hydrolysed by a variety of reagents both acid and alkaline. Lippich [1908] obtained a 92 % yield of leucine by heating its hydantoin for 24 hours with "fuming" hydrochloric acid. Dakin [1920] found that a 70 % yield of hydroxyproline was obtained when its hydantoin was boiled for 12 hours with a large excess of 10 % barium hydroxide solution. From experiments carried out in this laboratory, it appears that the respective times for complete hydrolysis of benzylhydantoin by 50 times its weight of constant boiling hydrochloric, hydrobromic and hydriodic acids are 20–30, 6–7 and 1–2 hours. Whilst these reagents are suitable from many points of view, they cannot, for reasons already given, be generally employed.

As has been mentioned, Harries and Weiss obtained glycine from hydantoin by heating the latter with commercial ammonium sulphide for 2 hours at 150°. These authors however omit to state the yield of amino-acid obtained. Since the use of ammonium hydroxide or ammonium sulphide (the sulphide being present presumably to protect the amino-acid in alkaline solution from atmospheric oxidation) has obvious advantages in the ready removal of the hydrolytic reagent and the isolation of the amino-acid, experiments were undertaken to determine the most suitable conditions for the hydrolysis of hydantoins by such means. From the results obtained, it appears that a suitable strength of reagent is about 6 % ammonium sulphide or 3 % ammonium hydroxide, and that whilst ammonium sulphide gives a practically theoretical yield, ammonium hydroxide under the same conditions gives only a 75–85 % yield of the amino-acid. Another very interesting fact was gleaned from these experiments. During the later stages of the hydrolysis of benzylhydantoin by 6 % ammonium sulphide solution, some colourless crystalline material separated. From its melting-point and other characteristics, this compound appeared to be phenylalanine anhydride. So far as we are aware the formation of this compound under such conditions has not been described before and it is being further investigated.

For purposes of presentation, the experimental work has been divided into the following sections:

(1) The action of aqueous ammonium sulphide at 100° on unsaturated hydantoins.

(2) The action of aqueous ammonium sulphide at 58° on unsaturated hydantoins.

(3) The action of sodium hydrogen sulphide at 100° on unsaturated hydantoins in (*a*) aqueous solution and (*b*) alcoholic solution.

(4) The action of hydrogen sulphide at 100° on unsaturated hydantoins in pyridine solution.

(5) The hydrolysis of reduced hydantoins by aqueous solutions of (*a*) ammonium sulphide and (*b*) ammonium hydroxide.

The successful use of hydrogen sulphide as a reducing agent for the purpose above described is novel and the possibility of the application of the reaction to other unsaturated substances of somewhat similar constitution suggests itself. It has already been found, for example, that hydrogen sulphide will reduce α-acetamidocinnamic acid and benzalcreatinine to phenylalanine and benzylcreatinine respectively. These and other extensions of the method will be discussed in a subsequent paper. Finally, the further possibility of the replacement of hydrogen sulphide by organic sulphides, *e.g.* thiolacetic acid or cysteine in such reactions is being investigated.

EXPERIMENTAL.

1. *The action of aqueous ammonium sulphide at 100° on unsaturated hydantoins.*

Benzylhydantoin and phenylalanine from benzalhydantoin. Benzalhydantoin (6·5 g.) was heated at 100° in a closed vessel for 64 hours with 80 ml. of commercial ammonium sulphide (16 %). The resulting solution was evaporated to dryness and treated with 200 ml. of boiling water and filtered. The filtrate was again evaporated to dryness, taken up in 200 ml. of hot water and re-filtered. The combined insoluble material consisting mainly of sulphur was extracted repeatedly with hot water and filtered. The united filtrates were concentrated until crystals of phenylalanine began to form on the surface. 100 ml. of 95 % alcohol were added, and the phenylalanine was allowed to crystallise out overnight. The amino-acid was collected and recrystallised from dilute alcohol. A second crop was obtained by concentrating the mother-liquor and adding more alcohol. This was collected in its turn and recrystallised. The total yield of amino-acid was 3·1 g. It melted at 263°, and contained 8·40 % N. Erlenmeyer [1893] gives M.P. 263°. $C_9H_{11}O_2N$ requires N 8·48 %.

The mother-liquors from which the phenylalanine separated were concentrated, acidified with hydrochloric acid and boiled for a few minutes. A white crystalline substance separated and this on recrystallisation from dilute alcohol melted at 188°; this was identified by M.P. and mixed M.P. with benzylhydantoin made from phenylalanine and potassium cyanate. On treating the two preparations separately with cold fuming nitric acid and adding cold water 4-benzyl-3-nitrohydantoin was obtained in each case. Both nitrohydantoins melted at 208° after recrystallisation from alcohol, and a mixture of the two melted at the same temperature. The benzylhydantoin obtained amounted to 2·2 g. and contained 14·67 % N. $C_{10}H_{10}O_2N_2$ requires N 14·74 %. These results showed that the reduction of the benzalhydantoin was practically quantitative.

p-*Methoxyphenylalanine and* p-*methoxybenzylhydantoin from* p-*methoxybenzalhydantoin.* In a similar manner to that described above, *p*-methoxybenzalhydantoin (1·0 g.) gave *p*-methoxyphenylalanine (0·5 g.; 50 %) melting at 235° and containing 7·25 % N. $C_{10}H_{13}O_3N$ requires N 7·18 %.

On acidification of the mother-liquor with hydrochloric acid and boiling, the corresponding hydantoin separated on cooling. On recrystallisation it melted at 182·5° and contained 12·64 % N. $C_{11}H_{12}O_3N_2$ requires N 12·73 %. The hydantoin prepared from *p*-methoxyphenylalanine and potassium cyanate melted at 181°, whilst a mixture of the two preparations melted at the same temperature. The yield of the hydantoin was 0·28 g.

Tyrosine from p-*hydroxybenzalhydantoin.* *p*-Hydroxybenzalhydantoin (2·65 g.) [*cf.* Boyd and Robson, 1935] was heated with 30 ml. of ammonium sulphide at 100° for 72 hours. On cooling, the mixture became semisolid owing to separation of the tyrosine. The crystals of amino-acid were collected and the remainder of the amino-acid was isolated as in the previous experiments. After recrystallisation from water and drying, the tyrosine weighed 0·75 g., contained 7·69 % N, and melted at 309° when heated rapidly, 300° when heated slowly. Wheeler and Hoffman [1911] state that the melting-point of synthetic tyrosine is "indefinite" and varies for the same specimen from 295 to 340° according to the rate of heating. $C_9H_{11}O_3N$ requires N 7·74 % The yield was 32 % of the theoretical.

Benzylthiohydantoin and benzylhydantoin from benzalthiohydantoin. Benzalthiohydantoin (2·4 g.) was heated with a mixture of 50 ml. of alcohol and 100 ml. of commercial ammonium sulphide at 100° in a closed vessel for 72 hours. The liquid was evaporated to dryness and the residue was taken up in dilute sodium hydroxide and filtered. The solution was made strongly acid by the addition of dilute hydrochloric acid and boiled for some minutes. The crystalline precipitate which separated on the solution cooling was recrystallised from hot water. It melted at 180°; after a further recrystallisation from water, at 185°. Johnson and Nicolet [1913] quote 185°. It contained 13·86 % N. $C_{10}H_{10}ON_2S$ requires N 13·60 %. It weighed 1·66 g., equivalent to 68 % of the theoretical.

Benzylthiohydantoin (1·0 g.) was boiled for one hour with 10 ml. of 50 % chloroacetic acid. The solution was then diluted with water and cooled. The hydantoin was collected and recrystallised from hot water. It melted at 189° and contained 14·60 % N. The yield was 0·67 g. or 73 % of theory.

p-*Methoxybenzylthiohydantoin and* p-*methoxybenzylhydantoin from* p-*methoxybenzalthiohydantoin.* *p*-Methoxybenzalthiohydantoin (2·0 g.) was heated with 50 ml. of 16 % ammonium sulphide for 72 hours at 100°. The reduced hydantoin was isolated as in the case of benzylthiohydantoin. It weighed 1·24 g., equivalent to 62 % of the theoretical. It melted at 215° and contained 11·73 % N. $C_{11}H_{12}O_2N_2S$ requires N 11·86 %. The preparation of this substance has not been recorded hitherto.

p-Methoxybenzylthiohydantoin (0·6 g.) was boiled with 10 ml. of 50 % chloroacetic acid for 1 hour and the *p*-methoxybenzylhydantoin was isolated as in the case of the benzylhydantoin. It weighed 0·41 g., melted at 182·5° and contained 12·84 % N.

2. *The action of aqueous ammonium sulphide at 58° on unsaturated hydantoins.*

Benzylhydantoin from benzalhydantoin. Benzalhydantoin (2·0 g.) was heated with a mixture of 50 ml. of 16 % ammonium sulphide solution and 50 ml. of alcohol for 240 hours at 58° in a closed bottle. The solution was evaporated to dryness, the residue boiled with 200 ml. of water and the mixture filtered. The insoluble sulphur remaining on the filter was extracted twice with hot water. The combined filtrates were acidified with hydrochloric acid, boiled with a little charcoal and filtered. The filtrate was concentrated and the benzylhydantoin allowed to crystallise out for 3 days. It was collected and washed with a little cold water. The dry hydantoin weighed 1·88 g. The volume of mother-liquor was about 25 ml. and since the solubility of benzylhydantoin is of the order of 1 g. per 1000 ml., it may be assumed that at least 0·03 g. remained in solution. The total yield was therefore 1·91 g. equivalent to 95 % of the theoretical value. The product melted at 187° and contained 14·63 % N. It is probable that a shorter period of heating the benzalhydantoin with the ammonium sulphide would suffice.

A similar experiment in which 50 ml. of ammonium sulphide without alcohol were used gave a yield of 51 % of the theoretical figure after 1 week at 58°. The insolubility of the benzalhydantoin in ammonium sulphide at 58° however was such that it was not completely dissolved until the sixth day. The reduction was therefore retarded by the insolubility of the unreduced substance.

p-*Hydroxybenzylhydantoin from* p-*hydroxybenzalhydantoin.* *p*-Hydroxybenzylhydantoin was prepared in a similar manner to that described for benzylhydantoin, the period of heating being extended to 2 weeks. The product melted at 254° and contained 13·69 % N. The yield was 90 % of the theoretical. $C_{10}H_{10}O_3N_2$ requires N 13·59 %.

p-*Hydroxybenzylthiohydantoin from* p-*hydroxybenzalthiohydantoin.* *p*-Hydroxybenzylthiohydantoin prepared from *p*-hydroxybenzalthiohydantoin as described for benzylhydantoin melted at 209–210°, and contained 12·78 % N. $C_{10}H_{10}O_2N_2S$ requires N 12·62 %. Johnson and Nicolet [1913] quote 211° as the M.P. of a specimen of *p*-hydroxybenzylthiohydantoin made by the action of ammonium thiocyanate and acetic anhydride on tyrosine.

3. *The action of sodium hydrogen sulphide at 100° on unsaturated hydantoins.*

(*a*) *In aqueous solution.*

Benzylhydantoin from benzalhydantoin. Benzalhydantoin (2·0 g.) was treated with the equivalent amount (0·4 g.) of sodium hydroxide in 30 ml. of water. The bottle containing the solution was immersed in ice and the solution saturated with hydrogen sulphide, the hydantoin being precipitated thereby. The bottle was sealed and heated at 100° for 15 hours. It was then cooled, again saturated with hydrogen sulphide and finally re-heated at 100° for a further 16 hours. The solution was then evaporated to dryness *in vacuo*. The residue was treated with hot water and filtered from the insoluble sulphur. The filtrate, made strongly acid by the addition of hydrochloric acid, was boiled for 10 minutes. After cooling and keeping overnight 1·0 g. of pure benzylhydantoin separated. It melted at 188° and contained 14·66 % N. The mother-liquor was concentrated, made neutral to litmus by the addition of sodium hydroxide and boiled with 0·5 g. of potassium cyanate for 30 minutes to re-convert the amino-acid, produced by hydrolysis, into carbamido-acid. The liquid was then made strongly acid with hydrochloric acid and boiled for 10 minutes. A further crop of 0·59 g. of benzylhydantoin separated when the liquid cooled. It melted at 189° and contained 14·60 % N. If allowance be made for the solubility of benzylhydantoin in the 250 ml. of mother-liquor on the assumption that one part dissolves in 1000 ml. of water, the total yield of benzylhydantoin was 1·84 g. or 92 % of the theoretical value. Part of this (32 %) however consisted of amino-acid reconverted into hydantoin. The reduction was therefore practically quantitative.

A second experiment was carried out in which the heating period was only 16 hours. Examination of the resulting product showed that only partial reduction had been effected.

(*b*) *In alcoholic solution.*

Benzylhydantoin from benzalhydantoin. Benzalhydantoin (2·0 g.), sodium hydroxide (5 ml. of 10 % aqueous solution), water (25 ml.) and alcohol (60 ml.) were mixed and saturated with hydrogen sulphide at 0–5°. The mixture was heated in a closed vessel at 100° for 16 hours, re-saturated with hydrogen sulphide at 0–5° and again heated for 44 hours longer. The resulting solution was evaporated to dryness *in vacuo* and the residue taken up in 100 ml. of hot water. The hot solution was filtered and the insoluble sulphur repeatedly extracted with boiling water and filtered. The combined filtrates were acidified strongly with hydrochloric acid, boiled with a little charcoal and filtered hot. The filter was washed twice with hot water. The combined filtrates were concentrated to 150 ml. *in vacuo* and allowed to cool overnight. 1·32 g. of benzylhydantoin, M.P. 188°, were obtained. After addition of 0·15 g. as a correction for the amount of material remaining in solution, the total yield amounted to 1·47 g. or 73 % of the theoretical.

A similar experiment carried out in 90 % alcohol instead of 60 % alcohol gave a total yield, on the same basis, of 1·60 g. of benzylhydantoin, or 78 % of the theoretical.

Further experiments were carried out using in each case 2·0 g. of benzalhydantoin dissolved in 100 ml. of absolute alcohol, to which was added (1) 0·6 g. of sodium hydrogen sulphide (1 mol.) or (2) 0·3 g. of sodium hydrogen sulphide respectively. The cold solution was saturated with hydrogen sulphide, heated for 16 hours at 100°, cooled, re-saturated with hydrogen sulphide and again heated for 16 hours at 100°. The experiment with 1 mol. of sodium hydrogen sulphide yielded 1·5 g. of reduced product, or 75 % of the theoretical. That with 0·5 mol. of sodium hydrogen sulphide yielded 1·62 g. of the required product or 80 % of the theoretical figure.

Finally, an experiment was carried out using 1·8 g. of benzalhydantoin, 2 ml. of 20 % sodium hydroxide solution and 100 ml. of absolute alcohol. The other conditions were the same as in the two preceding experiments. The yield of pure reduced product was here 1·59 g. or 87 % of theory.

It may be noted that solutions of benzylhydantoin which have been saturated at a higher temperature and then kept at room temperature do not attain equilibrium for several days. The solubility figure used above refers to solutions in pure water. The actual yields were probably a little higher than the values given.

p-*Hydroxybenzylhydantoin from* p-*hydroxybenzalhydantoin*. *p*-Hydroxybenzalhydantoin (2·0 g.) was dissolved in sodium hydroxide (2·0 ml. of 20 %) and alcohol (100 ml.). The solution was saturated at 0° with hydrogen sulphide, heated at 100°, cooled, re-saturated with hydrogen sulphide and again heated at 100° for 16 hours. After being cooled, the solution deposited 0·80 g. of pure *p*-hydroxybenzylhydantoin which melted at 256°. Wheeler and Hoffman [1911] quote 257–258°. A further 0·60 g. was obtained in the usual way after removal of sulphur from the solution. The yield was thus 70 % of the theoretical. The material contained 13·62 % N. $C_{10}H_{10}O_3N_2$ requires N 13·59 %.

Experiments carried out with larger proportions of sodium hydrogen sulphide or sodium hydroxide yielded an oily by-product.

4. *The action of hydrogen sulphide at 100° on unsaturated hydantoins in pyridine solution.*

p-*Methoxybenzylhydantoin from* p-*methoxybenzalhydantoin*. *p*-Methoxybenzalhydantoin (2·0 g.) was dissolved in pyridine (20 ml.) to which concentrated ammonia (1 ml.) had been added. The mixture was saturated at 0–5° with hydrogen sulphide and then heated at 110° in a closed vessel for 48 hours. The solution was cooled to 5°, re-saturated with hydrogen sulphide and again heated under the same conditions for a further 18 hours. The solution was evaporated to dryness *in vacuo*, the residue taken up in boiling water and the whole filtered to remove the insoluble sulphur. The latter was boiled out repeatedly with fresh quantities of hot water, the suspension being filtered each time. The combined filtrates were concentrated to 250 ml. and cooled. 1·62 g. of *p*-methoxybenzylhydantoin, melting at 182° and containing 12·80 % N, was thus obtained. On concentrating the mother-liquors a further 0·3 g. of the required material was obtained. The yield amounted to 95 % of the theoretical figure.

Benzylhydantoin from hydantoin and benzaldehyde without intermediate isolation of benzalhydantoin. Benzaldehyde (5·5 g.), hydantoin (4·5 g.), pyridine (10 ml.) and diethylamine (10 drops) were heated to 98° for 42 hours in a closed bottle. The mixture was then cooled, more pyridine (20 ml.) added, and the solution was saturated with hydrogen sulphide at −5°. It was thereafter heated for 66 hours at 98°. The benzylhydantoin was isolated as in the preceding experiments. It weighed 4·1 g. or 48 % of the theoretical.

5. (*a*) *The hydrolysis of reduced hydantoins by aqueous ammonium sulphide.*

Phenylalanine from benzylhydantoin. Benzylhydantoin (2·0 g.) was dissolved in a mixture of 10 ml. of concentrated ammonia (sp. gr. 0·880) and 90 ml. of water. The solution was saturated with hydrogen sulphide at 0°. The vessel containing the resulting mixture was then closed and heated for 5 days at 100°. At the end of this time the solution was clear except for a small quantity of white flocculent matter. It was filtered, the filtrate concentrated until the amino-acid began to separate and finally treated with 50 ml. of alcohol. Pure phenylalanine crystallised out in well-formed plates which were filtered off and washed with dilute alcohol. It weighed 1·4 g., melted at 262° and contained 8·39 % N. A second crop was obtained by concentrating the mother-liquor and treating it again with alcohol. This contained 8·35 % N and weighed 0·3 g. The total yield was, therefore, 1·7 g., which was practically theoretical.

The flocculent material removed from the solution by filtration before isolating the amino-acid melted at 292°, and was evidently phenylalanine anhydride. Phenylalanine anhydride was found by Erlenmeyer and Lipp [1883] to melt at 290–291°. The amount isolated in the above experiment weighed 0·03 g.

5. (*b*) *The hydrolysis of reduced hydantoins by aqueous ammonium hydroxide.*

An exactly similar experiment to the preceding was carried out but omitting the saturation of the solution with hydrogen sulphide. In this case the deposit found in the solution after the period of heating was dark brown in colour indicating that oxidation had taken place to a certain extent. The yield of amino-acid isolated was 1·41 g., equivalent to 80 % of the theoretical. It contained 8·37 % N.

SUMMARY.

Reduction of unsaturated hydantoins, obtained by condensing aromatic aldehydes with ordinary hydantoin and with 2-thio-3-acetylhydantoin respectively, is readily effected quantitatively by heating them at 100° with solutions of sulphides for 24–72 hours. For this purpose aqueous or alcoholic solutions of ammonium sulphide, sodium hydrogen sulphide or sulphides of organic bases may be employed. The accompanying hydrolysis which occurs at 100° in the presence of water may be avoided by using anhydrous or almost anhydrous solutions of ammonium sulphide or sodium hydrogen sulphide in alcohol, or of hydrogen sulphide in pyridine or by heating the unsaturated hydantoin with aqueous ammonium sulphide at a lower temperature (58°) for a longer period (7–14 days). Some alcohol is usually added to the aqueous ammonium sulphide to increase the solubility of the unsaturated hydantoin.

In general a cleaner product is obtained by this method than by those hitherto employed, and it is especially applicable in cases where the use of hydriodic acid or of sodium amalgam is precluded by the instability of other parts of the molecule.

Hydrolysis of the reduced product to the amino-acid may be carried out in conjunction with the reduction process or separately. Dilute solutions of ammonium sulphide give a quantitative yield of pure amino-acid from the reduced hydantoin in 120 hours at 100°. Dilute ammonium hydroxide solutions are almost equally satisfactory but the presence of the sulphide appears to inhibit the darkening (? oxidation) of the solution during the process of hydrolysis.

A small amount of material separates during the course of hydrolysis by ammonium sulphide. This seems to consist of amino-acid anhydride.

REFERENCES.

Boyd and Robson (1935). *Biochem. J.* **29**, 542.
Dakin (1920). *J. Biol. Chem.* **44**, 518.
Erlenmeyer (1893). *Liebig's Ann.* **275**, 1.
—— and Lipp (1883). *Liebig's Ann.* **219**, 179.
Harington (1926). *Biochem. J.* **20**, 300.
Harries and Weiss (1903). *Liebig's Ann.* **327**, 372.
Johnson and Nicolet (1913). *Amer. Chem. J.* **49**, 197.
Lamb and Robson (1931). *Biochem. J.* **25**, 1231.
Lippich (1908). *Ber. deutsch. chem. Ges.* **41**, 2957.
Wheeler and Hoffman (1911). *Amer. Chem. J.* **45**, 368.

LXIV. THE SYNTHESIS OF INDOLE-3-ALDEHYDE AND ITS HOMOLOGUES.

BY WILLIAM JOHN BOYD AND WILLIAM ROBSON.

From the Department of Physiology, University of London King's College.

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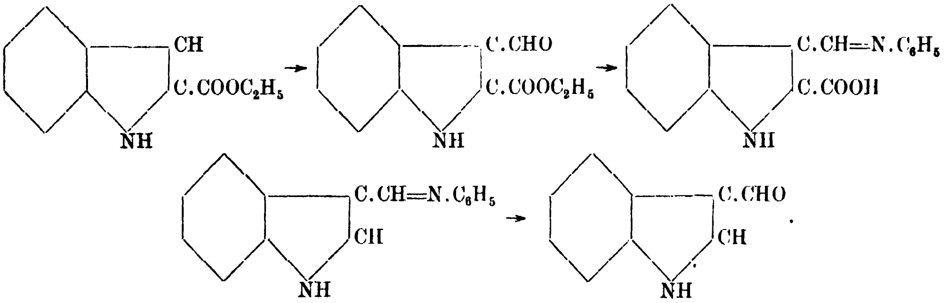
ONE of the many major difficulties encountered in the synthesis of tryptophan is that connected with the preparation of the parent substance, indole-3-aldehyde. Despite the exceedingly poor yields which Ellinger [1906] and Ellinger and Flamand [1908] obtained on synthesising this latter compound from indole, surprisingly few attempts have been made either to improve the yield or to evolve new methods of making it. The only one, indeed, worthy of mention is that of Majima and Kotake [1922] who employed a modified Grignard reaction on indole, using anisole instead of the customary ether as a solvent. The method, whilst it gives a primary yield of aldehyde higher than that obtained by Ellinger, suffers from the drawback that it is not applicable [Robson, 1924] with the same success to other derivatives of indole.

Preliminary experiments having already indicated that the modifications which may be successfully introduced into the Wheeler-Hoffman hydantoin synthesis of phenylalanine and tyrosine [Boyd and Robson, 1935, 1, 2] are applicable with even greater advantage to the synthesis of tryptophan from indole-3-aldehyde, it was decided to re-examine the problem of the preparation of the latter compound and its homologues with the view of making them more readily available.

The investigation followed two lines. The first had for its basis the modification of Ellinger's original method. On repeating the synthesis of indole-3-aldehyde according to that author it was noticeable that at the end of the reaction the resulting mixture still smelled strongly of chloroform. This fact in conjunction with the knowledge that Ellinger recovered unchanged half of the initial indole led to an examination of the quantities of the reacting substances used in the synthesis. It was at once evident that a much better yield of the aldehyde was to be expected if the quantity of hydroxide employed were considerably increased. An actual experiment proved this to be the case, the yield of aldehyde immediately obtainable being raised from the original figure of 9 % to one of 31 % of the figure demanded by theory. In addition, whilst 3-chloroquinoline is present as before in the steam distillate from the reaction mixture, it is accompanied by only insignificant amounts of unchanged indole. The same procedure applied to 2-methylindole and 5-methylindole gave the corresponding aldehydes in yields higher than those previously obtained by Ellinger's method.

In describing the synthesis of 7-methylindole-3-aldehyde, which has not been previously reported, mention must be made of the preparation of 7-methylindole. The first attempt to obtain this compound was made by Raschen [1887] along the lines of Fischer's classical method. For some reason, not apparent from his paper, Raschen did not succeed in decarboxylating the 7-methylindole-2-carboxylic acid. Following much the same procedure as Raschen, Kruber [1926] was able to isolate a small quantity of 7-methylindole and, moreover, evolved a method of isolating it from coal tar. In repeating Kruber's synthesis, the present writers have introduced modifications at certain stages. It has been found, for example, that ring closure is best effected by dissolving the pyruvic acid-*p*-tolylhydrazone in cold alcohol saturated with hydrochloric acid. When the same reaction is attempted with a hot alcoholic solution a second compound in addition to ethyl 7-methylindole-2-carboxylate is formed. Analysis of this substance indicates that it possesses the formula $C_{25}H_{20}O_7N_3$. It contains an amide grouping, the corresponding acid having the formula $C_{25}H_{19}O_8N_2$. Its structure is being further investigated. Kruber's method has been further improved in the last stage. Here, as has been reported previously in other cases, a much higher yield of the indole is obtained by heating the ammonium salt instead of the free carboxylic acid.

The second part of the investigation included several attempts to synthesise indole-3-aldehyde and its homologues on entirely new lines. Of these, only one merits description at the present stage. 2-Carbethoxyindole, by means of the modification of the well-known Gatterman hydrogen cyanide method introduced by Adams and Levine [1923], gave the corresponding 3-aldehyde in excellent yield. The ester aldehyde so obtained was hydrolysed without difficulty to the acid, the sodium salt of which in hot aqueous solution reacted with aniline, the anil thus formed being precipitated on carefully neutralising the cold solution.



Decarboxylation of the latter compound should yield the anil of indole-3-aldehyde. Attempts, however, to carry out this reaction were not altogether satisfactory owing to formation of tar. The best yields were obtained by heating the substance in liquid paraffin at 220° and precipitating the decarboxylated anil from solution therein by the addition of light petroleum. The compound thus obtained gave the required aldehyde on warming with water acidulated with a few drops of acetic acid. In the case of indole-3-aldehyde the yield approximated to 40–50 % but in the case of the 5-methyl and the 7-methyl homologues it was exceedingly poor.

During the course of this part of the investigation an attempt was made to synthesise 2-methylindole-3-aldehyde from 2-methylindole by the Adams-Levine method referred to above. It has already been shown by Barger and Ewins [1917] that on submitting 2-methylindole to the original Gattermann technique, the aldehyde is obtained in very good yield. On applying the method of Adams and Levine, however, we found that the product obtained was by no means homogeneous, but that it consisted of two fractions, one being easily soluble in hot alcohol whilst the other was practically insoluble in that medium. Further examination showed that the soluble fraction consisted of the required 2-methylindole-3-aldehyde and that the insoluble compound was the tri(2-methylindolyl)-

methane, originally obtained by Fischer and Pisbor [1923] by heating a mixture of 2-methylindole and 2-methylindole-3-aldehyde with alcoholic potash.

In reporting this work, the authors have to thank Mr W. R. Boon for valuable help in the preparation of the required 5-methylindole.

EXPERIMENTAL.

Indole-3-aldehyde. Indole (20 g.) was dissolved in a mixture of chloroform (150 ml.) and 96 % alcohol (400 ml.) contained in a 2 l. flask fitted with a rubber stopper carrying a reflux condenser, a stirrer and a dropping funnel. The mixture was kept gently boiling over an electric hot plate and stirred while a solution of potassium hydroxide (250 g.) in water (300 ml.) was gradually dropped in over a period of 4–5 hours. The mixture was boiled for 30 minutes after the last addition of potassium hydroxide. When the contents of the flask had cooled, the potassium chloride was collected on a Büchner funnel and washed with alcohol. The combined filtrate and washings were then steam-distilled, the receiver being changed when the chloroform and most of the alcohol had passed over. The distillation was continued for 30 minutes after the distillate was no longer turbid owing to the presence of 3-chloroquinoline.

The hot aqueous liquid in the flask was decanted from the tarry residue and set aside to cool. The tarry material remaining was dissolved in the minimum quantity of hot alcohol, the alcoholic solution poured into a litre of hot water, the whole again boiled until the tarry globules had coalesced (an action which can be hastened by the addition of a little sodium chloride to the solution) and then filtered through a hot fluted filter-paper. The tar remaining on the filter was extracted yet another time in the same way. Aldehyde separated from all three aqueous solutions. This was filtered off and the combined filtrates amounting to some 2·5–3·0 litres were concentrated to about 300 ml. This concentrated solution on cooling yielded a further crop of aldehyde. The total yield of aldehyde thus obtained amounted to 7·5 g. (31 % of theory). The crude material melted at 194° and after recrystallisation from water at 198° (*cf.* Ellinger and Flamand, 195°). It contained 9·57 % N. C_9H_7NO requires N 9·66 %.

2-Methylindole-3-aldehyde. 2-Methylindole (13 g.) gave, by the above method 8 g. of 2-methylindole-3-aldehyde (51 % of theory).

5-Methylindole-3-aldehyde. By the same procedure, 20 g. of 5-methylindole yielded 6·5 g. of 5-methylindole-3-aldehyde (27 % of theory).

Pyruvic acid-o-tolylhydrazone. *o*-Toluidine (107 g.) was slowly added to a litre of hydrochloric acid (sp. gr. 1·16), cooled to 0°. A semi-crystalline mass of the hydrochloride separated out. Sodium nitrite (69 g.) dissolved in 150 ml. of water was slowly added, the temperature being maintained at zero by means of a freezing mixture. Stannous chloride (580 g.) dissolved in 500 ml. of hydrochloric acid and cooled to zero was next added. A white precipitate separated out. The mixture was left overnight in a cool place and next day the white precipitate was filtered off and well pressed. It was then shaken with 600 ml. of 25 % sodium hydroxide solution and 800 ml. of ether, and the ethereal layer was separated. The extraction with ether was repeated three times and the combined extracts were washed with water and then shaken with a slight excess of 1 % hydrochloric acid. The aqueous layer was separated and the ether was again washed twice with 100 ml. of dilute acid. To the combined acid solutions a solution of pyruvic acid in water equivalent to the *o*-tolylhydrazine present was added slowly with stirring. The yellow crystalline hydrazone began to separate at once and finally the mixture became semi-solid. It was filtered by suction on a Büchner funnel, and the solid was washed with a little water, air-dried and crystallised from hot 80 % alcohol. It melted at 157° (Raschen gives 158°). Yield 82·2 % of the theoretical.

2-Carbethoxy-7-methylindole. The pyruvic acid-*o*-tolylhydrazone (60 g.) was dissolved in cold absolute alcohol (500 ml.) which had been previously saturated with dry hydrogen chloride. The solution was kept at room temperature overnight and then poured into 5 litres of cold water. After standing overnight the precipitated material had become hard and was collected and dried at 100°. The brown amorphous product weighed 58 g. It was distilled at atmospheric pressure in a current of superheated steam and yielded 27 g. of light yellow 2-carbethoxy-7-methylindole which was somewhat impure as it melted below 100°. After crystallisation from hot 80 % alcohol it gave 15 g. of pure ester (M.P. 128°). The material in the mother-liquor was precipitated by the addition of water, collected, dried and extracted with hot light petroleum. In this way a further 3 g. of pure ester was obtained. The yield of pure ester from 60 g. of the pyruvic acid-*o*-tolylhydrazone was thus 18g. or 28 % of the theoretical yield. On decarboxylation of the impure uncrystallisable material, however, pure 7-methylindole corresponding to a further 8 % was obtained, making the total yield of ester 36 % of the theoretical value.

When the ester of the pyruvic acid-*o*-tolylhydrazone was used instead of the acid, no improvement in yield or purity of 2-carbethoxy-7-methylindole was found. The hydrazone ester was prepared by dissolving 50 g. of the hydrazone acid in 500 ml. of absolute alcohol, adding 25 ml. of concentrated sulphuric acid and heating the mixture under reflux for 30 minutes. The mixture was then poured into several litres of water, the precipitate which formed was collected and crystallised from 80 % alcohol. The yield was practically theoretical. The product melted at 61° (Raschen gives 61°, Kruber 62°).

When the solution of the pyruvic acid-*o*-tolylhydrazone in absolute alcohol previously saturated with hydrogen chloride was kept overnight and then heated on the water-bath for an hour before being poured into water, no improvement in yield or purity was obtained.

When pyruvic acid-*o*-tolylhydrazone was dissolved in absolute alcohol and the solution was then saturated with dry hydrogen chloride the yield was much less, and another crystalline substance was formed in considerable quantity. This appears to be an amide of high molecular weight. It was formed under the following conditions. *o*-Tolylhydrazone of pyruvic acid (80 g.) was dissolved in absolute alcohol (500 ml.) and dry hydrogen chloride was passed in. After a time the flask was cooled to prevent the alcohol from boiling away, and the saturation of the alcohol was completed. The solution was kept at room temperature overnight, and next day a white crystalline deposit was found at the bottom of the flask. This substance, of which about 5 g. were present, was very insoluble in ether, benzene, petroleum, cold alcohol and cold water. It was recrystallised from hot water and then melted with decomposition at 283° and evolved ammonia on being warmed with dilute sodium hydroxide. On acidifying the alkaline solution with hydrochloric or sulphuric acid, an acidic substance was precipitated which melted after recrystallisation from water at 235°. It was more soluble in water than the parent substance and dissolved in sodium bicarbonate solution with evolution of carbon dioxide.

Elementary analysis of these substances showed them to be an amide and the corresponding acid of compositions corresponding respectively to the formulae $C_{25}H_{20}O_7N_3$ and $C_{25}H_{19}O_8N_2$. (Found for amide: C, 63·9; H, 4·0; N, 8·83 %. For acid: C, 63·6; H, 4·0; N, 5·9, 6·0 %.)

The substances did not give a coloration with Ehrlich's reagent or with glyoxylic acid and concentrated sulphuric acid.

7-Methylindole-2-carboxylic acid. The corresponding ester was hydrolysed by boiling under a reflux with 6 % alcoholic KOH for 30 minutes. The alcoholic solution was then poured into water, the alcohol distilled off and the solution acidified. The 7-methylindole-2-carboxylic acid which separated was filtered off and recrystallised from dilute alcohol. It formed fine needles melting at 173°. Raschen gives 171° and Kruber 170°. The yield was quantitative. The substance contained 7·94 %N. $C_{10}H_9O_2N$ requires N 8·00 %.

7-Methylindole. Although Raschen failed originally to obtain the indole by melting 7-methylindole-2-carboxylic acid, Kruber obtained it in this way. In the present case the ammonium salt was used. The acid was dissolved in dilute ammonium hydroxide and the solution evaporated to dryness on the water-bath. The dry ammonium salt was transferred to a small distilling flask and carefully heated in a metal-bath to 240° at which temperature it melted and evolved ammonium carbonate which condensed in the side tube of the flask and was expelled by warming. On further heating the indole distilled as a water-clear liquid which solidified to a white mass. The yield was 62 % of the theoretical. Crystallisation of the indole from alcohol gave white plates melting at 78°. After recrystallisation from light petroleum, it melted at 82°. Since Kruber has reported a melting-point of 85°, the product was twice recrystallised from light petroleum and the melting-point redetermined. It was still 82°. After careful sublimation of the product in a test-tube, it was still the same. Commercial 7-methylindole from coal-tar also melted at 82° before and after recrystallisation from light petroleum. A mixture of the synthetic and coal-tar products also melted at 82°. (Found: C, 82·54; H, 6·98 %. C_9H_9N requires C, 82·39; H, 6·92 %.)

The picrate, prepared by adding aqueous picric acid solution to a hot saturated aqueous solution of the indole, separated as the solution cooled as a crimson precipitate. It crystallised from light petroleum in fine needles, which melted at 172° (*cf.* Kruber, 175°).

In working up impure 7-methylindole-2-carboxylic ester which could not be purified by crystallisation it was found convenient to convert it into crude ammonium salt and to decarboxylate it as already described. The indole was then readily recovered reasonably pure by steam distillation. It melted at 78° on recrystallisation from alcohol.

7-Methylindole-3-aldehyde. This substance was prepared in exactly the same way as indole-3-aldehyde. The yield from 20 g. of 7-methylindole was 7–8 g. It crystallised in gritty prisms of a sandy colour or in plates and melted at 202°. It contained 8·89 % N. $C_{10}H_9ON$ requires N 8·81 %.

3-Chloro-8-methylquinoline. This substance, which has not been described hitherto, was obtained in the steam distillate of the reaction mixture obtained in the Tiemann-Reimer preparation of 7-methylindole-3-aldehyde. Since there was little unchanged 7-methylindole present, the chloromethylquinoline crystallised out from the aqueous distillate. The yield from 20 g. of 7-methylindole was 5·0 g. It crystallised in long needles from dilute alcohol melting at 55°. Found 19·79 % Cl. $C_{10}H_8NCl$ requires Cl 20·00 %.

2-Carbethoxyindole-3-aldehyde. 2-Carbethoxyindole (19 g.), zinc cyanide (17 g.) and 300 ml. of ether which had been distilled over sodium were placed in a flask fitted with (*a*) an inlet tube dipping below the surface of the ether, (*b*) an outlet tube connected through a sulphuric acid trap to the outside air and (*c*) an efficient stirrer. Dry hydrogen chloride was passed into the ether for

3 hours, the reaction flask being kept cold by an ice-bath. After 2 hours, the ice-bath was removed and one of warm water substituted so that the ether just boiled. After a further 30 minutes the reaction was considered complete. The 2-carbethoxyindoleiminochloride was deposited as a yellowish white substance on the sides of the flask. Next morning the ether was decanted off, and the inside of the flask and its solid contents were washed with ether. The imino-compound was then boiled with water and the aldehyde which separated was filtered off and crystallised from dilute alcohol. 15 g. of pure product melting at 187° (188–189° after recrystallisation from chloroform) were obtained. A further crop of 3 g. of crude product was isolated from the mother-liquor. 3·5 g. of unchanged carbethoxyindole were recovered from the ether. The 2-carbethoxyindole-3-aldehyde contained 6·49 % N. $C_{12}H_{11}O_3N$ requires N 6·45 %.

2-Carboxyindole-3-aldehyde anil. 3·0 g. of indole-3-aldehyde-2-carboxylic ester were warmed with 1·5 equiv. of dilute alcoholic sodium hydroxide for 15 minutes. 1·4 g. of aniline were added to the hot solution with stirring. The liquid was cooled and carefully neutralised to Congo red with dilute hydrochloric acid, whereupon the acid anil was precipitated. The yield of anil was practically quantitative. It melted at 239° (raised to 244–245° on recrystallisation from dilute alcohol). It crystallised in yellow needles and contained 10·52 % N. $C_{16}H_{12}O_2N_2$ requires N 10·61 %.

Indole-3-aldehyde anil. 1·5 g. of indolealdehyde-2-carboxylic acid anil were suspended in 15 ml. of medicinal paraffin and heated to 210°. Gas was evolved and the substance gradually passed into solution. After evolution of gas had ceased, the addition of light petroleum to the solution precipitated the decarboxylated product. This was filtered off, washed with light petroleum and recrystallised from chloroform and finally from light petroleum. It melted at 123° and contained 12·61 % N. $C_{15}H_{12}N_2$ requires N 12·73 %. A certain amount of tar was formed, but the yield was estimated at approximately 50 % of the theoretical.

0·5 g. of ordinary indole-3-aldehyde was heated with 0·7 g. of aniline in a test-tube. The product was crystallised from alcohol and recrystallised from 50 % alcohol. It melted at 122·5°. Majima and Kotake [1925] quote 126–127° as the melting-point of the product made by a different method.

Treatment of the anil with a very slight excess of dilute acetic acid sufficed to liberate the aldehyde.

2-Carbethoxy-7-methylindole-3-aldehyde. The preparation of this substance from 2-carbethoxy-7-methylindole followed exactly the preparation of 2-carbethoxyindole-3-aldehyde as described above. The yield was similar, but a small quantity of another product was obtained which was less soluble in alcohol. This has not been further investigated. After recrystallisation from alcohol the product melted at 189°, and contained 6·09 % N. $C_{13}H_{13}O_3N$ requires N 6·04 %.

2-Carboxy-7-methylindole-3-aldehyde anil. This substance was prepared from the ester-aldehyde (or from the acid-aldehyde) as described for the anil of 2-carboxyindole-3-aldehyde. It melted at 255° with decomposition. The yield was reduced by the formation of an insoluble yellow compound which melted at 245°, and could not be decomposed with acid to regenerate the aldehyde.

7-Methylindole-3-aldehyde anil. The preparation of this substance from the preceding compound by decarboxylation followed closely that of the anil of indole-3-aldehyde. It was also formed by fusing 7-methylindole-3-aldehyde and aniline together as described for the lower homologue. Both preparations melted at 128° and gave the aldehyde on treatment with very slight excess of acetic acid. The anil contained 11·82 % N. $C_{16}H_{14}N_2$ requires N 11·97 %.

2-Methylindole-3-aldehyde (*by the modified Gattermann method*). A mixture of 2-methylindole (15 g.), zinc cyanide (17 g.) and 150 ml. of dry ether was treated with dry hydrochloric acid gas (*cf.* the preparation of 2-carbethoxyindole-3-aldehyde above) first for 2·5 hours at 0° and finally for 30 minutes at 40°. The highly pigmented mass which had separated during the reaction was filtered off, washed several times with ether and then warmed with hot water. Gradually an almost colourless crystalline mass began to separate. After standing some time, this was filtered off and washed several times with water and dried. The dry product was triturated with hot alcohol which dissolved only a fraction of it. While still hot, the mixture was filtered and water was added to the filtrate until crystallisation commenced. When the mixture was cold, the crystals were filtered off and dried. They weighed 3·5 g. They had M.P. 201° (Barger and Ewins give 202–203°) and contained 8·9 % N. This fraction of the product was therefore 2-methylindole-3-aldehyde.

For purposes of purification, the alcohol-insoluble fraction of the product was dissolved in pyridine and reprecipitated with benzene, and was thus obtained almost colourless. M.P. 319°. It contained 10·62 % N. $C_{28}H_{25}N_3$ requires N 10·4 %. These characteristics showed that this fraction was undoubtedly the tri-(methylindolyl)methane obtained originally by Fischer and Pisbor [1923]. Approximately 10 g. of it were obtained by the above procedure.

SUMMARY.

Ellinger's original method of synthesising indole-3-aldehyde has been modified so that an increased yield of the product is obtained in one operation and the necessity of recovering unchanged indole is avoided. Similarly improved yields of 2-methylindole- and 5-methylindole-3-aldehyde have also been obtained by the modified method. Modification of the synthesis of 7-methylindole has led to increased yields of that substance; moreover, the synthesis of 7-methyl-3-aldehyde, which has not been recorded previously, is described. Details are given of an alternative method of synthesising indole-3-aldehyde.

Part of the work described above was done during the tenure of a Berridge Scholarship awarded to one of us (W. J. B.) by the Delegacy of the University of London King's College. The cost of the materials used was defrayed partly from a grant from the Royal Society to one of us (W. R.).

REFERENCES.

Adams and Levine (1923). *J. Amer. Chem. Soc.* **45**, 2373.
Barger and Ewins (1917). *Biochem. J.* **11**, 58.
Boyd and Robson (1935, 1). *Biochem. J.* **29**, 542.
—— —— (1935, 2). *Biochem. J.* **29**, 546.
Ellinger (1906). *Ber. deutsch. chem. Ges.* **39**, 2520.
—— and Flamand (1908). *Z. physiol. Chem.* **55**, 8.
Fischer and Pisbor (1923). *Ber. deutsch. chem. Ges.* **56**, 2313.
Kruber (1926). *Ber. deutsch. chem. Ges.* **59**, 2752.
Majima and Kotake (1922). *Ber. deutsch. chem. Ges.* **55**, 3862.
—— —— (1925). *Ber. deutsch. chem. Ges.* **58**, 2037.
Raschen (1887). *Liebig's Ann.* **239**, 223.
Robson (1924). *J. Biol. Chem.* **62**, 495.

LXV. THE EFFECT OF ASCORBIC ACID ON EXPERIMENTAL GOITRE.

By ALLAN WILLIAM SPENCE and ERIC FRANK SCOWEN[1].

From the Dunn Laboratories, St Bartholomew's Hospital, London.

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The experiments of Marine and his collaborators [Marine *et al.*, 1933] show that the development of simple goitre in rabbits, fed on a diet consisting chiefly of cabbage, can be prevented by the feeding of certain fresh plants and indicate that the plants in question contain an antigoitrigenic substance other than iodine. The authors suggest that the substance may be identical with hexuronic acid isolated by Szent-Györgyi [1928] from cabbage, orange juice and suprarenal cortex. They state however that iodine cannot be entirely excluded as a contributory antigoitrigenic factor in these experiments, and that final proof must await the production of thyroid involution by the use of a pure iodine-free preparation of hexuronic acid. The purpose of this paper is to show that in our experience hexuronic acid (ascorbic acid) has no involuting effect on thyroid hyperplasia produced in rabbits by means of cabbage feeding or on that produced in guinea-pigs by daily intraperitoneal injections of acid extract of anterior pituitary. Further, it will be seen that there is no definite evidence that a deficiency of ascorbic acid over a prolonged period has a goitrigenic action in guinea-pigs.

EXPERIMENTAL.

The effect of ascorbic acid on simple thyroid hyperplasia produced by cabbage feeding. The production of simple thyroid hyperplasia in rabbits fed on a diet consisting chiefly of cabbage was observed by Chesney *et al.* [1928], and confirmed by Marine *et al.* [1929] in the United States, by McCarrison [1931] in India and by Spence *et al.* [1933] in England. In the present experiment considerable thyroid enlargement and marked hyperplasia were produced in a batch of eight rabbits, aged 3 months and weighing 960–1537 g., by feeding them for one month on fresh cabbage in amounts equivalent to 75 Cals/kg. of body weight. The diet was supplemented with 35 g. of rolled oats three times weekly. The condition of the thyroid glands was observed surgically. The animals then continued with their cabbage diet, but two received daily by mouth 5 mg. and two 20 mg. of pure ascorbic acid, freshly dissolved in boiled distilled water. The remaining four served as controls. At the end of 20 days of this treatment the thyroids were again inspected surgically and also microscopically. The glands of two of those which had received ascorbic acid were perhaps slightly less hyperplastic than on the previous occasion, but this might well have been due to some diminution in the potency of the cabbage, as one of the controls had similarly regressed and two had not increased in size (Table I).

By the same means marked thyroid hyperplasia was produced in a second batch of eight rabbits, aged 3 months and weighing about 1500 g. The animals continued with their cabbage diet, but four of them received daily by mouth 50 mg. of ascorbic acid. The remaining four as controls received no medication.

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[1] Baly Research Scholar.

At the end of 3 weeks there was no diminution in the size of the thyroid glands, and histologically all showed marked hyperplasia with no evidence of involution (Table I).

Table I. *The effect of ascorbic acid on simple thyroid hyperplasia in rabbits. (Period: 3 weeks.)*

							Final condition of thyroid	
No.	Sex	Age (months)	Initial wt. g.	Previous condition of thyroid	Daily dose of ascorbic acid g.	Final wt. g.	Size or wt. g.	Histology (hyperplasia)
158	M	5	960	+ +	5	1067	+	Moderate
161	F	5	1295	+ +	5	1448	+ +	Marked
179	F	4	1310	+	20	1392	+ −	Moderate
182	F	4	1310	+	20	1325	+	,,
134	F	6½	1537	+ +	Nil	1655	+ +	Marked
143	M	6	1348	+	,,	1580	+ −	Moderate
176	M	4	1117	− +	,,	1268	+ −	Marked
177	M	4	1032	− +	,,	1193	− +	Moderate
222	M	3	1475	+	50	1982	283	Marked
223	M	3	1875	+ +	50	2322	580	,,
225	F	3	1675	− +	50	1975	224	Moderate
232	F	3	1620	+ −	50	2172	432	Marked
229	F	3	1400	+ +	Nil	1842	477	,,
233	F	3	920	− ?	,,	1315	286	Moderate
234	M	3	1410	+ +	,,	1935	650	Marked
235	M	3	1602	− +	,,	1800	297	,,

+ + = More than twice normal size, marked hyperaemia.
+ = About twice normal size, marked hyperaemia.
+ − About 1½ times normal size, moderate hyperaemia.
− + = Slightly enlarged, slight hyperaemia.
− ? = Possibly slightly enlarged, possibly slight hyperaemia.

(The average weight of the normal thyroid of an adult rabbit is about 150 mg.)

Table II. *The effect of ascorbic acid on thyroid hyperplasia produced in guinea-pigs by anterior pituitary extract.*

						Condition of thyroid		
No.	Sex	Initial wt. g.	Daily dose of ascorbic acid (subcutaneous) mg.	Daily dose of anterior pituitary extract (intra-peritoneal) ml.	Final wt. g.	Wt. mg.	Histology (hyper-plasia)	Wt. of supra-renals mg.
156	F	230	25 (vi. 28–vii. 9)	1 (vii. 2–9)	250	73	Marked	164
157	F	240	25 (,,)	1 (,,)	242	60	,,	160
158	M	290	25 (,,)	1 (,,)	320	85	,,	180
159	M	270	25 (,,)	1 (,,)	307	53	Moderate-marked	162
160	M	298	25 (,,)	1 (,,)	318	68	,,	218
161	M	265	50 (vi. 28–vii. 8)	1 (vii. 2–8)	292	55	Marked	160
162	M	240	50 (,,)	1 (,,)	244	37	,,	177
163	F	227	50 (,,)	1 (,,)	222	68	,,	230
164	F	287	50 (,,)	1 (,,)	267	73	,,	230
165	F	215	50 (,,)	1 (,,)	197	35	,,	154
166	F	240	Nil	1 (vii. 2–9)	281	55	,,	140
167	F	210	,,	1 (,,)	257	78	,,	160
168	M	277	,,	1 (,,)	292	54	,,	211
169	M	272	,,	1 (,,)	212	124	,,	214
170	M	265	,,	1 (,,)	285	80	,,	205
171	M	275	,,	1 (vii. 2–8)	321	63	,,	171
172	M	267	,,	1 (,,)	245	91	,,	158
173	F	200	,,	1 (,,)	237	57	,,	233
174	F	250	,,	1 (,,)	290	50	,,	222
175	F	247	,,	1 (,,)	212	57	,,	232

The effect of ascorbic acid on thyroid hyperplasia produced by anterior pituitary extract. Twenty guinea-pigs, ten males and ten females, weighing 200–300 g., were fed on a non-goitrigenic diet containing fresh lettuce three times weekly. They were divided into two groups in which the sexes were equally distributed. Both groups received daily intraperitoneal injections of 1 ml. of acid extract of Organon's anterior pituitary powder, prepared after the method of Loeb and Bassett [Scowen and Spence, 1934]. In one group, however, five received in addition 25 mg. and five 50 mg. of ascorbic acid daily subcutaneously. The administration of ascorbic acid was started 4 days before treatment with anterior pituitary extract was begun. It has been shown by numerous observers, including Aron [1929; 1930], Collip [1933] and Loeb and Friedman [1931], that the initial hyperplasia produced by anterior pituitary extract regresses and involutes to the colloid state in spite of continued administration. In some cases the hyperplasia may reach its maximum in one or two weeks. So that this regression should not be a complicating factor and vitiate the results, the animals were sacrificed after receiving anterior pituitary for 7–8 days. The thyroid glands of both groups, *i.e.* those which received ascorbic acid and those which did not, all showed macroscopically marked hyperaemia and histologically marked hyperplasia. There was no significant difference in the weights of the suprarenals in either group (Table II).

The effect of a diet deficient in ascorbic acid on the thyroid gland of guinea-pigs. Should ascorbic acid be an antigoitrigenic substance, a deficiency of it might

Table III. *The condition of the thyroid gland in acute and chronic scurvy* (*guinea-pig*).

(Diet: crushed oats, bran and autoclaved milk.)

							Condition of thyroid	
No.	Sex	Initial wt. g.	Daily dose of ascorbic acid mg.	Duration of experiment days	Condition of animal	Final wt. g.	Size or wt. mg.	Histology
7619	M	267	Nil	22	Acute scurvy	340	Not enlarged	Normal
7620	F	218	,,	22	,,	340	,,	,,
7621	M	255	,,	22	,,	242	,,	,,
7622	F	265	,,	22	,,	250	,,	,,
7623	M	265	,,	22	Acute scurvy; pneumonia	225	,,	,,
7624	F	280	,,	22	Acute scurvy	300	,,	,,
7613	F	255	0·25–0·5	39	Chronic scurvy	245	,,	,,
7614	M	260	,,	44	,,	245	,,	Marked hyperplasia
7615	M	270	,,	39	,,	265	,,	Slight hyperplasia
7616	F	290	,,	44	,,	280	,,	Moderate hyperplasia
7617	M	255	,,	44	,,	220	,,	Normal
7618	M	260	,,	33	,,	230	,,	,,
7761	M	285	,,	41	,,	362	51	,,
7762	F	295	,,	41	,,	230	50	,,
7763	F	275	,,	41	,,	247	62	Slight hyperplasia
7764	M	295	,,	41	,,	297	55	,,
7765	M	305	,,	41	,,	315	51	Normal
7809	M	295	,,	26	,,	330	42	,,
7650	F	250	5	44	Normal	370	38	,,
7651	F	266	5	44	,,	370	41	,,
7652	M	275	5	44	,,	442	38	,,
7653	F	250	5	44	,,	372	41	,,
7654	F	255	5	44	,,	425	40	,,
7655	M	285	5	44	,,	465	40	,,

be expected to bring about hyperplasia of the thyroid gland in a considerable percentage of animals. To test this hypothesis we examined the thyroid glands of guinea-pigs which were maintained on scorbutic diets as suggested by Dr S. S. Zilva of the Lister Institute, where he kindly carried out the experiments for us. Twenty-four guinea-pigs weighing 200–300 g. were fed on a scorbutic diet consisting of a daily ration of 15–20 g. of a mixture of 1 part by volume of crushed oats, 2 parts by volume of bran and about 40 ml. of reconstituted dried milk, autoclaved at 120°. They were divided into three groups. The first group consisting of six animals was fed on this diet for 22 days, and at the end of this period all were in a condition of acute scurvy. The second group consisting of twelve guinea-pigs received the diet for periods varying from 26 to 44 days, and was maintained in a condition of chronic scurvy by the daily administration of ascorbic acid in doses varying from 0·25 to 0·5 mg., the amount being regulated by the animal's weight and daily condition. The third group of six guinea-pigs received the diet for 44 days, but each animal was fully protected against scurvy by a daily dose of 5 mg. of ascorbic acid. At the end of their respective periods the animals were sacrificed. The thyroids of the six guinea-pigs with acute scurvy were normal, both macroscopically and microscopically. The thyroids of the six guinea-pigs which were fully protected against scurvy were also normal. Of the twelve animals suffering from chronic scurvy, one showed marked, one moderate and three slight hyperplasia. There was no definite enlargement of the glands (Table III).

CONCLUSION.

In view of the antigoitrigenic effect of plant juices the consideration of the part played by vitamin C (ascorbic acid) in the aetiology of simple goitre is important. Contradictory results have been obtained in studying the effect on the thyroid gland of vitamin C deficiency. McCarrison [1919–20] found that guinea-pigs fed on a scorbutic diet consisting of crushed oats and autoclaved milk and deficient in essential salts developed thyroid enlargement, mainly as a result of congestion and haemorrhage. He does not state whether hyperplasia was present. In later experiments he found no thyroid enlargement in rats fed on a synthetic diet deficient in vitamin C but complete in other respects [McCarrison, 1930]. Harris and Smith [1928], on the other hand, observed a slight degree of thyroid hyperplasia in guinea-pigs in which experimental scurvy had been produced by means of a diet of alfalfa meal, wheat flour, oats and water.

In the experiment described above it has been seen that in acute scurvy there was no evidence of thyroid hyperplasia, but that in twelve guinea-pigs with chronic scurvy thyroid hyperplasia was present in a fairly marked degree in two and in a slight degree in three animals. We feel that this finding occurred in too small a percentage to have any definite significance. It has also been shown that the daily administration of large doses of ascorbic acid does not prevent the occurrence of thyroid hyperplasia in guinea-pigs receiving injections of anterior pituitary extract containing thyrotropic hormone, a finding which is not in agreement with the conclusions of Marine *et al.* [1934]. Neither has ascorbic acid any involuting effect on the hyperplastic goitres produced in rabbits by means of cabbage, which itself is rich in vitamin C. We are consequently of the opinion that ascorbic acid cannot be regarded as an antigoitrigenic substance.

SUMMARY.

1. The administration of ascorbic acid in daily doses of 5, 20 and 50 mg. for 3 weeks did not cause involution of hyperplastic thyroids produced in rabbits by means of cabbage.
2. The administration of ascorbic acid in daily doses of 25 and 50 mg. for 12 days did not prevent the occurrence or diminish the degree of thyroid hyperplasia in guinea-pigs which received at the same time 8 daily injections of thyrotropic hormone of the anterior pituitary.
3. In six guinea-pigs with acute scurvy, there was no evidence of thyroid hyperplasia; in twelve guinea-pigs with chronic scurvy there were varying degrees of hyperplasia in five animals, but there was no significant thyroid enlargement.
4. It is concluded that ascorbic acid is not an antigoitrigenic substance.

We wish to thank Dr A. Macbeth, of the Organon Laboratories, for supplies of anterior pituitary powder.

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REFERENCES.

Aron (1929). *Compt. Rend. Soc. Biol.* **102**, 682.
—— (1930). *Compt. Rend. Soc. Biol.* **103**, 145.
Chesney, Clawson and Webster (1928). *Bull. Johns Hopkins Hosp.* **43**, 261.
Collip (1933). *Lancet*, i, 1208.
Harris and Smith (1928). *Amer. J. Physiol.* **84**, 599.
Loeb and Friedman (1931). *Proc. Soc. Exp. Biol. Med.* **29**, 172.
McCarrison (1919–20). *Ind. J. Med. Res.* **7**, 633.
—— (1930). *Ind. J. Med. Res.* **18**, 577.
—— (1931). *Ind. J. Med. Res.* **18**, 1311.
Marine, Baumann and Cipra (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 822.
—— —— and Rosen (1934). *Proc. Soc. Exp. Biol. Med.* **31**, 870.
—— —— Webster and Cipra (1933). *J. Exp. Med.* **57**, 121.
Scowen and Spence (1934). *Brit. Med. J.* ii, 805.
Spence, Walker and Scowen (1933). *Biochem. J.* **27**, 1992.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.

LXVI. A STILL FOR CONCENTRATION UNDER REDUCED PRESSURE.

By COLIN JOHN OWEN RHONABWI MORRIS.

From the Biochemical Department, Lister Institute, London.

(*Received January 20th, 1935.*)

In the course of work with protein hydrolysates, it was necessary to concentrate large volumes of acid liquids at a fairly low temperature. The acidity of the solutions to be concentrated precluded the use of metal parts in the apparatus, and the solutions were prone to froth during concentration under reduced pressure in a simple still.

The apparatus to be described obviated these difficulties. Fig. 1 shows the layout of the still. The distillation vessels consist of two 2 l. long-necked flasks A_1, A_2 (only one of these is shown in the figure), connected by the short tubes B_1, B_2, to the arms C_1, C_2, of the stillhead D. The arms C_1, C_2, are of 18 mm. internal diameter and permit the free flow of the vapour to the enlarged upper portion of the tube D. This is provided with an external water-jacket E, and an internal cooler F, which extends to the bottom of the receiver G. F is located centrally in D by three glass projections (not shown). The receiver G is cooled externally by the overflow water from F which is distributed over its surface by the tubes H. In order to empty the receiver without dismantling the apparatus, it is provided with two tubes K_1, K_2, connected by a two-way tap to the water-pump. During normal distillation, the pump communicates with K_2, but when it is desired to empty the receiver, the pressure in the system is

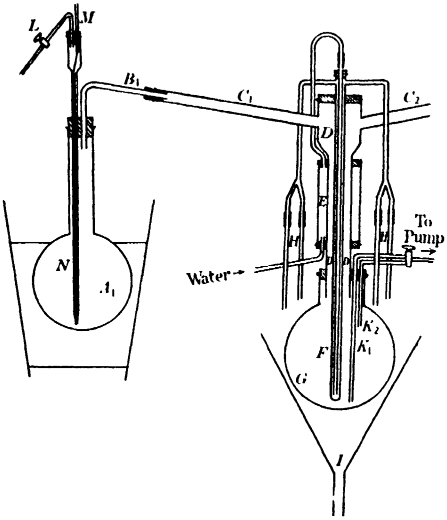

Fig. 1.

increased slightly and the tap turned to communicate with K_1; the contents of G can then be sucked over into a suitable receiver placed between the pump and the still.

With foaming liquids, a modified air-leak has been found very useful. The liquid to be concentrated enters continuously at L and is mixed with air from the very fine capillary M. The foam passes down the tube N, which is drawn out somewhat at its lower end. It is found that this device prevents both bumping and excessive foaming in the distillation flasks. In the case of a non-foaming liquid, an ordinary air-leak can be used, and the feed L then passes directly into the distillation flask A.

The distillation flasks and the condensing system are clamped independently, and the rubber sleeves between B and C permit small relative movements. The condensing system is also supported on the funnel I by three cork blocks (not shown).

The distillation flasks are heated in baths consisting of buckets heated by gas-rings. The large volume of liquid makes loss by evaporation unimportant at the low temperatures used.

The advantages of the still are as follows:

(1) It is cheap and simple to construct and operate.

(2) Owing to the free passage afforded to the vapour and to the fact that it is cooled somewhat before it passes through the more constricted parts of the system, the efficiency is high. At a water-bath temperature of 50–55° and a pressure of 12 mm. it has a capacity of $1\frac{1}{2}$–2 l. per hour, depending on the liquid being concentrated.

(3) The central cooler minimises re-evaporation of the condensed liquid and the establishment of back-pressure.

(4) The liquid to be distilled can enter and the condensate can be removed from the system without a large increase in pressure.

(5) Frothing is minimised.

LXVII. THE INFLUENCE OF ANIMAL TISSUES ON THE OXIDATION OF ASCORBIC ACID.

BY COLIN ASHLEY MAWSON.

From the John Burford Carlill Laboratories, Westminster Hospital Medical School, S.W. 1.

(*Received January 30th, 1935.*)

IT has usually been considered that ascorbic acid cannot be kept under aerobic conditions except in the solid form unless in relatively strongly acid solution. Green [1933] suggested that under the conditions of p_H and oxygenation of living tissues the existence therein of reduced ascorbic acid might be explained by the presence of some anti-oxidative mechanism. St Huszák [1933] showed that a substance occurred in the suprarenal medulla which prevented the reduction of silver nitrate by the ascorbic acid of suprarenal medulla and cortex. This protective substance was precipitated by lead acetate. Further work by de Caro [1934] and by de Caro and Giani [1934] which appeared during the course of this investigation led to the conclusion that in most animal tissues a protective mechanism existed of the kind postulated by Green. This work showed that ascorbic acid in phosphate buffer (p_H 7·5) would remain unoxidised in presence of air for several hours if mixed with small amounts of liver, muscle, blood, ovary, nerve or suprarenal gland from normal or scorbutic guinea-pigs. The protective material could be extracted by phosphate buffers, trichloroacetic acid or alcohol and was not precipitated by lead acetate or mercury. Although extracts deprived of reduced glutathione by mercury treatment still possessed protective power, de Caro and Giani considered that traces of oxidised glutathione had escaped precipitation and would account for the protection, but they pointed out that in this case the substance responsible differed from that observed by St Huszák, which was precipitated by lead acetate.

In the present paper it has been shown that although no protective mechanism exists in the lemon apart from the acidity of the juice, small traces of animal tissues or tissue extracts will prevent the oxidation of ascorbic acid in $M/30$ phosphate buffer at p_H 7·4 when incubated at 37°. This protective effect is most powerful in the presence of whole tissues, but extracts filtered through Bechhold collodion membranes (0·7 μ pores) still contain the protective mechanism. Active extracts have been made from the liver, spleen, brain and muscle of rats.

The protective effect of tissues can be closely imitated by cysteine, cystine, H_2S and glutathione. Extracts treated with mercuric acetate retain only about 1/100 of their original activity and at the same time lose most of their glutathione, but by heating liver extract for one hour in a boiling water-bath the protective activity can be reduced to about 1/10 of its original value without any change in the glutathione content. Addition of iodoacetate has no effect on protection by liver extract and only slightly reduces protection by glutathione when present in excess. Quantitative experiments showed that far more glutathione, as the pure substance, was necessary to prevent the oxidation of ascorbic acid than could be found in extracts giving optimum protection, and dialysed extracts containing practically no reduced glutathione had exactly the same protective power as undialysed extracts. The various pure substances used did not give as high a degree of protection as liver extract, the presence of which, at optimum concentration, would usually preserve 90 % of the original concentration of ascorbic acid for an hour at 37° in $M/30$ phosphate (p_H 7·4).

Ascorbic acid is comparatively stable at 37° if dissolved in water twice redistilled from a glass apparatus, but the addition of minute traces of copper caused rapid oxidation. Ferrous and ferric ions catalysed the oxidation to a less extent, while mixtures of iron and copper caused oxidation to take place more rapidly than addition of either metal alone. It is considered likely that the rapid oxidation of ascorbic acid solutions made from ordinary laboratory distilled water is due to contamination with traces of copper and iron.

Copper in fairly high concentration catalysed the aerobic oxidation of glutathione, but iron had no appreciable effect, confirming the results of Pirie [1931]. Addition of iron to liver extracts did not reduce their power to protect ascorbic acid from oxidation until a concentration of 10^{-3} mg. per ml. was reached, and iron had little effect on the protective power of glutathione. A reduction in the protective power of glutathione was effected by 4×10^{-4} mg. Cu per ml., but that of liver extract containing the same amount of glutathione was only reduced by copper concentrations greater than 2×10^{-3} mg. per ml. The amounts of free copper and iron in the serum, and especially in the liver, are so large that without some anti-oxidative mechanism the oxidation of ascorbic acid would be very rapid indeed. Tompsett [1934] gives the copper content of blood serum as 2×10^{-3} mg. per ml. and Fowweather [1934] states that the iron content of serum is about $1{\cdot}6 \times 10^{-3}$ mg. per ml. At the copper concentration of serum a 0·005 % solution of ascorbic acid in glass-distilled water was almost completely oxidised in one hour without the addition of any iron at all.

It has not been found possible to decide upon the nature of the undialysable part of the protective mechanism, but several results suggest that in the fresh extract the effect is due to several substances, some of which can be removed by dialysis and some precipitated by mercury or trichloroacetic acid or by boiling, but whichever of these processes is used there is always sufficient of the protective mechanism left behind to be easily detectable. It seems unlikely that the protective effect can be wholly due to the same substance or group of substances in each case.

EXPERIMENTAL.

Ascorbic acid has been estimated by titration with 0·01 % 2:6-dichlorophenolindophenol [Harris and Ray, 1933] in 4 % trichloroacetic acid solution. Glutathione has been estimated in 4 % trichloroacetic acid solutions containing ascorbic acid by oxidation of the ascorbic acid with the dye, addition of starch and titration with $N/500$ iodine. Using mixtures of glutathione and ascorbic acid, it was found that these substances could be accurately estimated by this method.

Where tissues or tissue extracts containing "ascorbic acid" or glutathione have been used, appropriate corrections have been applied. Unless otherwise stated, reagents and extracts have been made up with water distilled through a tinned copper coil with a solid tin nozzle and this has been referred to as "ordinary" distilled water. All metallic salt solutions have been made up in glass-distilled water.

In an attempt to decide whether the reducing substance which occurs in normal animal tissues was identical with that of tumour tissue [Boyland, 1933], and whether these substances were indeed ascorbic acid, an investigation was made of the effect of exposure to a temperature of 37° on the reducing values of various tissues. It was found that whether the tissue were whole or minced, alone or in presence of phosphate ($M/30$, p_H 7·4), or with phosphate + glucose, the amount of reducing substance remained unchanged, at any rate for periods up to 4 hours at 37° (Table I). These results confirm a recent observation by Guha and Ghosh [1934] on normal rat tissues.

Table I. *"Ascorbic acid" in tissues incubated at 37°.*

Tissue 1 g. minced	Duration of incubation mins.	ml. dye	mg. "ascorbic acid" per g.
Rat liver	0	7·40	0·33
	90	7·77	0·35
Rat testes	0	7·52	0·34
	90	7·77	0·35
Rous chicken tumour	0	1·48	0·07
	60	1·32	0·06

Table II. *Ascorbic acid incubated at 37° with and without minced rat liver.*

All experiments had total volume of 4·5 ml. (phosphate $M/34$, p_H 7·4).

	Ascorbic acid alone				
Incubation time (hrs.) ...	0	½	1	2	24
ml. dye	3·82	0·30	0·32	0·20	0·16
ml. iodine ($N/500$)	0·10	0·10	0·10	0·10	0·10
	Liver alone				
Incubation time (hrs.) ...	0	½	1	2	24
ml. dye	4·31	4·30	4·54	4·28	2·14
ml. iodine ($N/500$)	2·05	2·22	2·13	2·08	1·63
	Ascorbic acid + liver				
Incubation time (hrs.) ...	0	½	1	2	24
ml. dye	8·07	8·25	8·11	8·14	5·55
ml. iodine ($N/500$)	2·18	2·14	2·07	2·20	1·78

Blank on reagents: 0·19 ml. dye, 0·10 ml. iodine.

The instability of ascorbic acid in air at p_H 7·4 is well known, so it seemed doubtful whether the reducing substance could really be ascorbic acid. In order to check this point ascorbic acid solution (B.D.H. commercial) was kept at 37° with and without minced rat liver tissue in presence of $M/30$ phosphate, p_H 7·4. Although the ascorbic acid without additions had almost completely disappeared in half an hour at 37°, the same amount of ascorbic acid added to 0·5 g. minced liver was not oxidised at all after 2 hours. Even after 24 hours a large proportion still remained, though autolytic changes probably made the estimations in the latter case somewhat unreliable (Table II, Fig. 1).

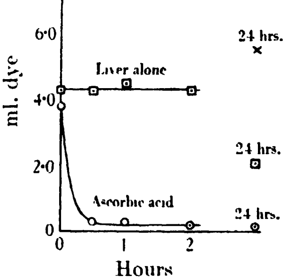

Fig. 1. Stabilisation of ascorbic acid by minced liver.

In this experiment, and in subsequent experiments of a similar nature, the iodine titre obtained subsequent to oxidation with 2:6-dichlorophenolindophenol has been assumed to represent glutathione, and it is evident that under the conditions of this experiment the glutathione content of the incubated tissue remained constant at 37° for at least 2 hours whether added ascorbic acid were present or not.

The stability of the reducing substance of liver, and the exact similarity of behaviour of ascorbic acid added to liver, are in such marked contrast to the rapid disappearance of ascorbic acid in absence of added tissue that this experiment tends to confirm the view that these substances are identical. The stability of the reducing substance (subsequently referred to as "ascorbic acid") is not confined to short periods of incubation at 37°, for if an animal was killed and kept at 4° for 24 hours, or if 5 % trichloroacetic acid extracts of the tissues were kept under similar conditions, the amount of "ascorbic acid" present did not decrease to any great extent.

Since tissues maintained their "ascorbic acid" content equally well whether minced or whole, the behaviour of extracts and suspensions was investigated. In these and subsequent experiments rat liver was generally used because of the ease with which it could be ground up and the large amount available in each animal, but similar results were obtained with other tissues. The suspensions were made by grinding the tissue with $M/30$ phosphate, p_H 7·5, and the extracts by centrifuging such suspensions for 15 minutes at 3000 r.p.m., pouring off, and re-centrifuging the supernatant liquid for the same time.

Both "ascorbic acid" and glutathione disappeared more rapidly in extracts than in suspensions, and both were more stable in the more concentrated preparations. In each case the "ascorbic acid" and glutathione occurring naturally in these preparations were estimated; no additions were made (Table III). The "ascorbic acid" in liver extracts and suspensions is more stable than the glutathione, but the decrease in amount of these substances during incubation does to some extent run a parallel course. A comparison was therefore made between the protective properties of rat liver extract and those of B.D.H. glutathione. In both cases an optimum concentration could be determined at which the maximum anti-oxidative effect was operative and the curves showing the variation of the protective effect with glutathione concentration were very similar in general appearance (Table IV, Fig. 2). In a series of ten experiments with centrifuged liver extracts the amount of liver necessary to give optimum protection varied considerably (from 0·13 mg. per ml. of ascorbic acid solution to 1·70 mg. per ml.), the mean value being 0·6 mg. per ml. The latter amount of rat liver would contain about $1{\cdot}5 \times 10^{-3}$ mg. glutathione, but the actual amount of reduced glutathione in the extracts was only of the order of 5×10^{-4} mg per ml. owing to the rapid oxidation of dilute solutions of liver glutathione.

Table III. *"Ascorbic acid" and glutathione in liver extracts and liver suspensions after various periods of incubation at 37°.*

Blanks on reagents subtracted.

		% remaining after x hours									
		"Ascorbic acid"					Glutathione				
Preparation	mg. tissue per ml.	0	½	1	2	4	0	½	1	2	4
*Suspension	200	100	103	105	101	87	100	97	96	90	87
*Extract	200	100	107	107	106	93	100	92	86	76	64
Suspension	100	100	99	99	98	87	100	99	98	71	48
Extract	100	100	93	86	80	68	100	70	52	32	14
*Suspension	5	100	87	87	76	51	100	36	30	28	29
*Extract	5	100	88	83	64	34	100	1·7	1·7	7·4	8·4
†Glutathione alone	1 mg. per ml.	—	—	—	—	—	100	57	30	Nil	Nil

* Mean of two experiments. † B.D.H. glutathione in $M/30$ phosphate, p_H 7·4.

Table IV. *Comparison of protective powers of centrifuged liver extract and glutathione.*

2 ml. liver extract (or 2 ml. glutathione solution) in $M/30$ phosphate p_{H} 7·4 + 2 ml. ascorbic acid in same buffer. Incubated in open tubes for one hour at 37°. Unincubated control 2·87 ml. dye. Blanks on reagents subtracted.

Liver dilution	1/40	1/400	1/2000	1/4000	1/8000	1/16,000	1/32,000
Containing mg. G.SH per ml.	3×10^{-2}	3×10^{-3}	6×10^{-4}	3×10^{-4}	$1{\cdot}5 \times 10^{-4}$	8×10^{-5}	4×10^{-5}
ml. dye	2·34	2·80	2·65	0·85	0·05	0	0
% of control	82	98	93	30	1·8	0	0
mg. B.D.H. glutathione per ml.	$1{\cdot}8 \times 10^{-1}$	$1{\cdot}8 \times 10^{-2}$	9×10^{-3}	$4{\cdot}4 \times 10^{-3}$	$1{\cdot}1 \times 10^{-3}$	$2{\cdot}2 \times 10^{-4}$	$2{\cdot}2 \times 10^{-5}$
ml. dye	1·58	1·78	1·17	0·74	0	0	0
% of contro	55	62	41	26	0	0	0

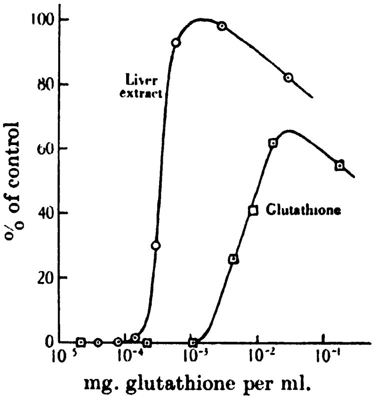

Fig. 2. Stabilisation of ascorbic acid by liver extract and glutathione.

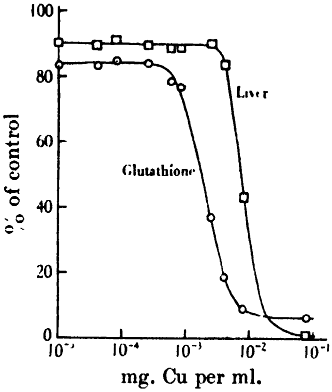

Fig. 3. Stabilisation of ascorbic acid by liver extract and glutathione, inhibition by copper.

The amount of B.D.H. glutathione necessary to give optimum protection was of the order of $3{\cdot}3 \times 10^{-2}$ mg. per ml. At the optimum liver concentration the protection afforded during 1 hour at 37° was usually about 90 %, but the protection given by glutathione seldom rose above 75 %. Nevertheless some of the pure glutathione might have been oxidised before addition to the ascorbic acid, although the glutathione and ascorbic acid solutions were both made in air-free phosphate and the tubes set up as rapidly as possible. Dr E. Boyland suggested that the commercial glutathione might contain traces of copper which

Table V. *Effect of copper on protective properties of liver extract and glutathione.*

Liver extract and B.D.H. glutathione (each 0·02 mg. G.SH per ml.) for 1 hour at 37° with ascorbic acid and $CuSO_4$. Unincubated control 11·75 ml. dye. Blanks on reagents subtracted.

mg. Cu per ml.	0	4×10^{-5}	8×10^{-4}	$2{\cdot}4 \times 10^{-3}$	4×10^{-3}	8×10^{-3}	8×10^{-2}
ml. dye with liver	10·53	10·48	10·45	10·62	9·88	5·08	0·10
% of control	90·0	89·3	89·1	90·5	84·2	43·3	0·85
ml. dye with G.SH	9·81	9·80	9·05	4·41	2·25	1·05	0·78
% of control	83·6	83·6	77·1	37·6	19·2	9·0	6·6

might act as a positive catalyst in the oxidation of ascorbic acid. Investigation of this suggestion led to several interesting results.

A rat liver extract was prepared so as to contain 0·10 mg. liver glutathione per ml., and the protective effect of this material against oxidation of added ascorbic acid was compared with that of a solution containing 0·10 mg. B.D.H. glutathione per ml. Various amounts of copper sulphate were added to each, and the mixtures incubated for 1 hour at 37° in a total volume of 5 ml. $M/30$ phosphate p_H 7·4, containing 0·5 mg. ascorbic acid (Table V, Fig. 3). The addition of copper has very similar inhibitory effects upon the protective properties of liver and glutathione, but the suppression of the protective effect of glutathione commences at a copper concentration of 4×10^{-4} mg. per ml., whereas in the case of liver extract containing the same amount of glutathione a copper concentration of $2{\cdot}3 \times 10^{-3}$ mg. per ml. is necessary. At the latter concentration the protection by glutathione is inhibited to the extent of 50 %.

A somewhat similar experiment was done using ferrous sulphate, in which the glutathione and liver concentrations were so arranged as to have equal anti-oxidative effects, and under these conditions ferrous sulphate had little effect on protection by glutathione but at concentrations higher than 10^{-3} mg. Fe^{++} per ml. the protection by liver was considerably decreased (Table VI).

Table VI. *Effect of Fe^{++} on anti-oxidative powers of liver extract and glutathione.*

2 ml. extract (1 part in 1000) or 2 ml. B.D.H. glutathione (0·05 mg. per ml.) for 1 hour at 37° with 2 ml. ascorbic acid. Unincubated control 4·17 ml. dye.

mg. Fe^{++} per ml.	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
ml. dye with liver	3·50	3·46	3·60	3·13	0·70	1·05
ml. dye with G.SH	3·53	3·62	3·54	3·44	2·72	3·53

These experiments suggested the possibility that the disappearance of ascorbic acid in aqueous solution might be connected with catalysis by heavy metals, and a preliminary experiment showed that ascorbic acid was remarkably stable in solution in water which had been twice re-distilled from an all-glass apparatus. Table VII gives the rates of oxidation in glass-distilled, ordinary distilled and London tap-water. The ordinary distilled water, which has been used throughout these experiments unless otherwise stated, was prepared by means of a tinned copper coil with a solid tin nozzle. It is quite evident from these results that something is present in ordinary distilled water and in tap-water which very greatly accelerates oxidation of ascorbic acid.

Table VII. *Oxidation of ascorbic acid in various types of water.*

3·7 mg. ascorbic acid in 20 ml. glass-distilled water. 1 ml. incubated with 4 ml. tap, ordinary distilled or glass-distilled water. Blanks on reagents subtracted.

Duration of incubation (mins.)		0	4	6	10	20	40
ml. dye	London tap-water	2·85	1·05	0·77	0·19	0·05	0·05
	Ordinary distilled water	3·33	2·56	2·12	1·66	0·33	0·00
	Glass-distilled water	3·38	3·39	3·76	2·95	2·74	2·40

The amount of copper which must be added to glass-distilled water in order to cause an appreciable increase in the rate of oxidation of ascorbic acid is less than 2×10^{-6} mg. Cu per ml. (Table VIII (*a*)). 2×10^{-4} mg. Fe^{++} or Fe^{+++} have a similar effect, but even at higher concentrations of iron the catalytic effect of this metal is not so great as that of copper. If copper and iron are present

Table VIII.

(*a*) *Influence of $CuSO_4$ on aerobic oxidation of ascorbic acid and glutathione.*

2 ml. ascorbic acid (or G.SH) + 2 ml. $CuSO_4$ incubated 1 hour at 37°.
Blanks on reagents subtracted.

mg. Cu per ml.	0	2×10^{-6}	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}	2×10^{-1}
ml. dye	4·48	3·16	3·06	2·46	0·11	0·08	0·04
Ascorbic acid: % of control	71	50	48	39	1·8	1·3	0·3
mg. Cu per ml.	0	6×10^{-7}	6×10^{-6}	6×10^{-5}	6×10^{-4}	6×10^{-3}	6×10^{-2}
ml. $N/500$ iodine	3·01	3·03	3·05	3·02	2·99	2·13	1·57
Glutathione: % of control	100	101	102	101	99	71	52

(*b*) *Influence of $FeSO_4$ on aerobic oxidation of ascorbic acid and glutathione.*

2 ml. ascorbic acid (or G.SH) + 2 ml. $FeSO_4$ incubated 1 hour at 37°.

mg. Fe^{++} per ml.	0	2×10^{-6}	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}	2×10^{-1}
ml. dye	4·48	3·95	4·31	3·66	3·39	1·95	1·58
Ascorbic acid: % of control	73	64	70	59	55	32	26
ml. $N/500$ iodine	2·77	2·73	2·71	2·72	2·76	2·76	2·77
Glutathione: % of control	99	98	98	98	99	99	99

(*c*) *Influence of $FeCl_3$ on aerobic oxidation of ascorbic acid.*

2 ml. ascorbic acid + 2 ml. $FeCl_3$ incubated 1 hour at 37°.

mg. Fe^{+++} per ml.	0	$1·8\times10^{-6}$	$1·8\times10^{-4}$	$1·8\times10^{-3}$	$1·8\times10^{-2}$	$1·8\times10^{-1}$
ml. dye	3·92	4·10	3·84	2·41	1·86	0·05
Ascorbic acid: % of control	72	75	71	44	34	0·1

(*d*) *Influence of $FeCl_3 + CuSO_4$ on aerobic oxidation of ascorbic acid.*

2 ml. metal solution + 2 ml. ascorbic acid incubated 1 hour at 37°.

mg. Cu^{++} per ml.	0	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}
mg. Fe^{+++} per ml.	0	4×10^{-4}	4×10^{-7}	4×10^{-4}	4×10^{-7}	4×10^{-4}	4×10^{-7}
ml. dye	3·92	0·04	0·10	0·61	1·19	1·09	2·43
Ascorbic acid: % of control	72	0·1	0·2	11	22	20	45

together, however, the catalytic effect is greater than that due to either metal alone (Table VIII (*b*), (*c*) and (*d*)). Fe^{++} has no effect on the rate of aerobic oxidation of glutathione, which is, however, increased by copper at concentrations greater than 6×10^{-4} mg. per ml. (Table VIII (*a*) and (*b*)). Addition of tin has no effect on the aerobic oxidation of either ascorbic acid or glutathione.

Since serum contains about 2×10^{-3} mg. Cu per ml. [Tompsett, 1934] and $1·6\times10^{-3}$ mg. Fe per ml. [Fowweather, 1934] it is evident that the relative stability of ascorbic acid in tissues cannot be explained by absence of a metallic catalyst, and we must look for a special protective mechanism. De Caro and Giani [1934] stated that cysteine and cystine both had effects similar to that of glutathione in the protection of ascorbic acid, and this has been confirmed. It has also been found that very minute traces of H_2S have a similar effect (Table IX). From the results with cysteine and cystine it seemed probable that the sulphydryl group played no part in the phenomenon, and this conclusion was supported by the observation that the protective power of liver was unaffected

Table IX. *Prevention of aerobic oxidation of ascorbic acid by cysteine, cystine and H_2S.*

4 ml. $M/30$ phosphate p_H 7·4 containing ascorbic acid and added compound, incubated 1 hour at 37°. Blanks on reagents subtracted.

mg. cysteine per ml.	$3{\cdot}7\times10^{-2}$	$3{\cdot}7\times10^{-3}$	$3{\cdot}7\times10^{-4}$	$3{\cdot}7\times10^{-5}$	$3{\cdot}7\times10^{-6}$
% of control	66	65	40	1·1	0
mg. cystine per ml.	$5{\cdot}2\times10^{-2}$	$5{\cdot}2\times10^{-3}$	$5{\cdot}2\times10^{-4}$	$5{\cdot}2\times10^{-5}$	$5{\cdot}2\times10^{-6}$
% of control	65	65	51	1·1	0
mg. H_2S per ml.	$1{\cdot}5\times10^{-2}$	$1{\cdot}5\times10^{-3}$	$1{\cdot}5\times10^{-4}$	$1{\cdot}5\times10^{-5}$	$1{\cdot}5\times10^{-6}$
% of control	108	90	82	20	1·8

by iodoacetic acid. Glutathione protection is only slightly reduced by considerable excess of iodoacetate (Table X).

Table X. *Effect of iodoacetate on protection of ascorbic acid by liver and glutathione.*

Blanks on reagents subtracted.

(*a*) Dilutions made from 10 ml. 1/4 liver extract + 1·0 mg. iodoacetic acid at 18° for 30 minutes or 10 ml. extract untreated under same conditions. Incubated 1 hour at 37° in $M/30$ phosphate p_H 7·4.

Liver dilution		1/40	1/400	1/4000	1/40,000
ml. dye	No iodoacetate	3·76	4·32	4·51	0·01
	Iodoacetate	3·62	4·38	4·35	0·03

(*b*) 2 ml. 1/1000 liver extract + 0·25 ml. iodoacetate + 2 ml. ascorbic acid. Incubated 1 hour at 37° in $M/30$ phosphate p_H 7·4. Control 4·00 ml. dye.

mg. iodoacetate per ml.	$1{\cdot}3\times10^{-1}$	$2{\cdot}5\times10^{-2}$	$2{\cdot}5\times10^{-3}$	$1{\cdot}5\times10^{-5}$
ml. dye	3·62	3·76	3·65	3·85

(*c*) 2 ml. ascorbic acid, 1 ml. glutathione (0·1 mg. G.SH), 1 ml. iodoacetate $M/30$ phosphate p_H 7·4. Incubated 1 hour at 37°. Control 3·40 ml. dye.

mg. iodoacetic acid	0	0·001	0·01	0·03	0·06	0·15	0·30
ml. dye	2·61	2·70	2·72	2·50	2·49	2·40	2·27

0·1 mg. glutathione is equivalent to 0·06 mg. iodoacetic acid.

It was found that if liver extract was kept in a boiling water-bath for one hour with the tube loosely corked its glutathione content remained practically unchanged but its protective power was reduced to one-tenth of normal. If reducing substances were removed from the extract by mercury precipitation [Emmerie and Van Eekelen, 1934] the glutathione content was considerably reduced and the protective power became only 1/100 that of untreated extract (Table XI). Trichloroacetic acid extracts of liver retained a large proportion of the protective properties of the untreated tissue.

Table XI. *Effect of boiling and mercury treatment on anti-oxidative properties of liver extract.*

2 ml. extract + 2 ml. ascorbic acid in $M/30$ phosphate p_H 7·4. Incubated 1 hour at 37°. Blanks on reagents subtracted.

Liver dilution added		1/20	1/200	1/2000	1/20,000	mg. G.SH per ml. 1/20
Ascorbic acid: % of control	Boiled	73	89	17	0	0·07
	Mercury-treated	96	59	1·5	0	0·01
	Untreated	83	95	99	0	0·07

The evidence presented up to this point tends to suggest that, although the effect of glutathione on the stability of ascorbic acid is very similar to that of liver extract, the effect of liver cannot wholly be accounted for by its glutathione content. Nor can any possible cysteine or cystine present be responsible as the amount necessary would be far too large. Since mannitol in minute traces is known to inhibit the aerobic oxidation of sulphite [Bigelow, 1898] this substance was tried, but neither mannitol nor inositol had any inhibitory effect on the aerobic oxidation of ascorbic acid. Indeed it seemed likely that the properties of liver extract depended upon the combined protective effect of traces of several reducing substances, until an experiment was carried out on the protective powers of dialysed and undialysed liver extracts. Suspensions of liver tissue in phosphate or distilled water were dialysed in collodion sacs or by means of cellophane membranes kindly supplied by the Cellophane Co., Ltd. During dialysis the liquid within the dialyser was stirred by means of a fine stream of nitrogen. Dialysis was continued for 3½–4½ hours against running tap-water, and then for one hour against 100 volumes of glass-distilled water. The suspension so obtained was centrifuged and was usually found to contain no detectable iodine-reducing substances. These extracts, however, retained unimpaired their power to prevent the aerobic oxidation of ascorbic acid. The dialysed extracts were compared with extracts which had been kept in stoppered tubes in the dialysis bath and which, though they usually lost a considerable proportion of their reduced glutathione, always retained a comparatively large amount of this substance. The facts that cysteine and cystine possess protective powers of the same order of magnitude and that glutathione loses little of this property in presence of iodoacetate suggest that a change from reduced to oxidised glutathione would have little effect on the protective power of this compound. In a series of ten experiments with dialysed materials, not all of which were quite free from reduced glutathione, no difference could be found between the protective powers of dialysed and undialysed extracts. The protocol of one experiment is given in Table XII.

Table XII. *Effect of dialysis on protective power of liver extract.*

1/10 extract dialysed 4½ hours in tap-water and 1 hour in glass-distilled water. Ascorbic acid in $M/30$ phosphate (p_H 7·5) made up with ordinary distilled water. 5 ml. dialysed extract required 0·12 ml. dye and 0·13 ml. $N/500$ iodine. 5 ml. undialysed extract required 2·09 ml. dye and 0·63 ml. iodine.

Blanks: 0·15 ml. dye; 0·13 ml. iodine.

1 ml. dye ≡ 0·050 mg. ascorbic acid. 1 ml. iodine ≡ 0·60 mg. glutathione.

2 ml. liver extract + 2 ml. ascorbic acid solution.

Liver dilution added ...	1/10	1/100	1/1000	1/3000	1/7000	1/10,000
Dialysed extract:						
G.SH (mg.)	0	0	0	0	0	0
ml. dye	2·53	2·77	2·30	0·19	0·15	0·18
Undialysed extract:						
G.SH (mg.)	6×10^{-2}	6×10^{-3}	6×10^{-4}	2×10^{-4}	9×10^{-5}	6×10^{-5}
ml. dye (corr.)	2·75	2·75	2·30	0·25	0·22	0·20

It was of interest to find whether any protective mechanism existed in vegetable material similar to that found in animal tissues, and for this purpose 10 ml. of lemon juice were brought to p_H 7·5 by addition of 5 ml. of dilute ammonia. 10 ml. of untreated juice were diluted to 15 ml. and 5 ml. of each sample diluted to 50 ml. with $M/30$ phosphate, p_H 7·5. 5 ml. portions were incubated at 37°, and it was found that the ascorbic acid in the neutralised material disappeared at the same rate as ascorbic acid in phosphate solution, while that in the diluted juice which had not been treated with ammonia was relatively stable. The p_H of the un-neutralised juice was less than 3·6 (Table XIII).

Table XIII. *Incubation of lemon juice.*

Blanks on reagents subtracted.

Time (mins.)		0	5	15	39	60	120	240
Untreated	ml. dye	5·83	5·74	5·57	4·99	4·02	2·47	0·79
	% of control	100	99	96	86	70	42	14
Neutralised	ml. dye	4·58	3·64	2·47	0·63	0·05	0·03	0·06
	% of control	79	62	42	11	0·9	0·5	1·0

It is evident, therefore, that the stability of ascorbic acid in the lemon is entirely due to the low p_H of the juice and not to any protective mechanism such as that found in animal tissues.

Discussion.

Although it has not been found possible to identify all the constituents of the mechanism which prevents the spontaneous oxidation of ascorbic acid in the animal body, it has been shown that glutathione is probably one of the factors involved. De Caro and Giani [1934] concluded that glutathione was the substance which caused stabilisation, but it cannot be wholly responsible for the phenomenon since liver extracts from which glutathione has been removed by prolonged dialysis retain the protective properties of fresh extract. The protective properties of boiled liver extract and extract which has been treated with trichloroacetic acid are likely to be due to their glutathione content, but the main part of the protective mechanism is of an undialysable nature. Although certain sulphydryl compounds when present in small traces have the property of preventing the oxidation of ascorbic acid, their effectiveness in this direction does not necessarily lie in the —SH group. De Caro and Giani found considerable differences in the protective properties of cysteine and cystine, although the curves were along the same general lines, but we found that equal amounts of these compounds had practically equal protective powers over wide ranges of concentration. This might be explained by a condition of equilibrium between cysteine and cystine, but this is made less likely by the fact that iodoacetate only slightly inhibits the protective properties of glutathione even when present in more than equimolecular proportions. Dickens [1933] has shown that iodoacetic acid condenses with sulphydryl compounds to give substances of the type $RS.CH_2.COOH$.

It is likely that a chain of protective mechanisms of a similar kind will be found to occur in animal tissues. Abderhalden [1934] states that tyrosine, 3:4-dihydroxyphenylalanine, and more especially adrenaline, are stabilised against oxidation by traces of ascorbic acid, and in a private communication Dr Heard states that work done in Toronto confirms Abderhalden's results.

The instability of aerobic solutions of ascorbic acid can be explained by the presence of small traces of copper and iron, which act as very powerful catalysts in the oxidation of ascorbic acid. The protective properties of tissue extracts and of the pure compounds investigated are probably due to their inhibition of metallic catalysts, which are present in body fluids in such a concentration that without a protective mechanism of some kind ascorbic acid could never exist in them at all.

The stability of the reducing substance of tumour tissue when in contact with tissue and the fact that its concentration in tissue is neither increased nor decreased by periods of incubation with or without glucose or phosphate are in such close agreement with the similar properties of ascorbic acid as strongly to suggest that these substances are identical. Before this can be stated with confidence, however, more biological and chemical work is necessary.

SUMMARY.

1. The ascorbic acid-like reducing substances of normal and tumour tissue are unaltered in amount by incubation of whole or minced tissue with or without phosphate or glucose for periods up to 4 hours at 37°.

2. Small amounts of animal tissues or tissue extracts prevent the aerobic oxidation of ascorbic acid solutions.

3. Glutathione, cysteine, cystine and H_2S prevent the aerobic oxidation of ascorbic acid, but protection by tissue extracts cannot wholly be explained by these facts.

4. Dialysed tissue extracts retain their anti-oxidative properties in full. These properties are also present, in reduced amount, in extracts which have been boiled or precipitated with mercuric acetate or trichloroacetic acid.

5. Ascorbic acid is relatively stable in glass-distilled water. Copper, and to a less extent iron, and especially mixtures of these two metals act as positive catalysts in the aerobic oxidation of ascorbic acid.

6. Lemon juice contains no protective mechanism other than the low p_H.

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REFERENCES.

Abderhalden (1934). *Fermentforsch.* **14**, 367.
Bigelow (1898). *Z. physikal. Chem.* **26**, 493.
Boyland (1933). *Biochem. J.* **27**, 802.
de Caro (1934). *Z. physiol. Chem.* **223**, 229.
—— and Giani (1934). *Z. physiol. Chem.* **228**, 13.
Dickens (1933). *Biochem. J.* **27**, 1141.
Emmerie and Van Eekelen (1934). *Biochem. J.* **28**, 1153.
Fowweather (1934). *Biochem. J.* **28**, 1160.
Green (1933). *Biochem. J.* **27**, 1044.
Guha and Ghosh (1934). *Nature*, **134**, 739.
Harris and Ray (1933). *Biochem. J.* **27**, 303.
St Huszák (1933). *Z. physiol. Chem.* **222**, 229.
Pirie (1931). *Biochem. J.* **25**, 1565.
Tompsett (1934). *Biochem. J.* **28**, 1544.

LXVIII. THE INFLUENCE OF ALKALI CATIONS ON THE FERMENTATION CAPACITY OF YEAST.

By ARTHUR LASNITZKI[1] AND EMERICH SZÖRÉNYI.

From the Biochemical Laboratory of the Cancer Research Institute at the University of Berlin.

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In a recently published communication [Lasnitzki and Szörényi, 1934] experiments on the influence of different cations on the growth of yeast cells were described. They showed that growth, under the experimental conditions used, was greatly increased by potassium and rubidium, and very little, if at all, by lithium, while sodium and caesium had an intermediate action. The influence of these five alkali cations thus agreed with their position in the Hofmeister series. A growth-promoting influence was also found for magnesium, while calcium, under the same conditions, had hardly any action.

We have been occupied with a second parallel series of investigations on the influence of alkali cations on the fermentation capacity of yeast. The object of these researches was to find out whether the energy metabolism of yeast cells, which must be considered as a necessary condition of growth, is increased by alkali cations and whether a similar order of action may be found in this case as in the activation of growth. It is these investigations which form the subject of this paper.

The influence of alkali salts on alcoholic fermentation has, like their influence on growth of yeast, up to the present time not been very systematically investigated. Harden [1917] found when using washed acetone-yeast and dried yeast that the activation of glucose fermentation in a medium containing phosphate by pyruvic acid or acetaldehyde only took place in the presence of potassium (or ammonium) and not at all or at the most to a slight degree in the presence of sodium. On the other hand Harden and Henley [1921], in confirmation of corresponding investigations of Meyerhof [1918], found that a surplus of potassium phosphate in the medium inhibited the fermentation of glucose and fructose by (unwashed) acetone-yeast or yeast-juice, and that other alkali salts (sodium and potassium sulphate, sodium and potassium chloride) in a high concentration acted in a similar manner. There is a doubt, however, whether the effects obtained can be ascribed to the cations. Contrary to the above authors the following workers used living yeast cells. Boas [1921] tested the action of lithium, sodium, potassium and ammonium nitrates (0·1 and 0·2 *M*) on the fermentation of sucrose by a suspension of brewer's yeast in pure water. The addition of the salts, with the exception of lithium nitrate, led to an increase of fermentation intensity, which, however, as a rule was near the limit of experimental error, and in no case was the increase more than 30 %. Also the difference in the action of the individual alkali salts was but little evident. Speakman *et al.* [1928] investigated the influence of sodium chloride on fermentation by brewer's yeast and found that weak concentrations somewhat accelerated whilst stronger concentrations inhibited. Since, however, the yeast cells under the experimental conditions used (wort as medium, and long duration of experiments) grew considerably the results were evidently not very clear. Finally Lindahl [1933; 1934], who worked under conditions similar to those of Boas, found that the fermentation of glucose by brewer's yeast was inhibited by lithium chloride, the amount of this inhibition being increased as the concentration of the salt was augmented, while the addition of potassium at the same time to a certain extent modified this inhibition. These results agreed with the findings in corresponding experiments upon the respiration of fertilised eggs of sea-urchins. Lindahl further stated that the oxidation of glucose and the dephosphorylation of hexosediphosphoric acid was inhibited by lithium chloride while the oxidation of glycerophosphoric acid and ethyl alcohol was accelerated.

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[1] At present at the Cancer Research Department, University of Manchester.

METHODS.

Alcoholic fermentation was measured by estimating the amount of carbon dioxide developed, Warburg's [1926] manometric method being used. The culture medium was similar to that used in our growth experiments, but in order to exclude the effect of respiration, fermentation was carried out under anaerobic conditions.

We proceeded as follows. 10 to 30 mg. of fresh baker's yeast was accurately weighed and shaken for about 10 minutes with 10 to 20 ml. of "suspension fluid". The suspension fluid had a similar composition to that used in the growth experiments. 0·1 g. diammonium hydrogen phosphate, 0·03 g. magnesium sulphate ($MgSO_4, 7H_2O$), 7·5 g. glucose, 0·75 g. asparagine, 0·15 g. tartaric acid, distilled water to 100 ml. The homogeneous cell suspension was centrifuged at a high speed for 5 minutes, the supernatant fluid poured off and the sediment diluted with suspension fluid so that the quantity of cells per ml. corresponded to 0·5–1·5 (usually 1·0) mg. of fresh yeast. 2 ml. of the suspension (kept constantly shaken) were measured into medium sized metabolism troughs (in one experiment 1 ml. in smaller troughs also). 1 ml. of "addition solution" (or 0·5 ml. respectively) was then added, and as control the same volume of distilled water. The addition solutions were 0·03 *M* solutions of lithium, sodium, potassium, rubidium and caesium chlorides. Having mixed these with the cell suspension in the proportion of 2:1, we had as in the growth experiments an alkali chloride concentration of 0·01 *M*. As regards the hydrogen ion concentration (p_H of the control medium about 3·1), it was probably near the upper limit of the zone which allows an optimum fermentation by living yeast. Before beginning the experiment the medium was saturated with nitrogen. The temperature employed was as a rule 21°, but in some cases a little higher. The readings were mostly made at intervals of 15 minutes. The time between the addition of the alkali chlorides and the first reading was about 1 to 1½ hours.

The experimental conditions in the fermentation experiments differed in three respects from those of the growth experiments: (1) much smaller quantity of cells; (2) shorter duration of experiments; (3) anaerobic conditions instead of the aerobic (or partial anaerobic) conditions in the growth experiments.

RESULTS.

Our results are expressed by the "fermentation quotient" which is defined as

$$Q_{CO_2} = \frac{\text{carbon dioxide developed, in } \mu l \text{ (0°, 760 mm. Hg)}}{\text{mg. yeast}^1 \times \text{time (in hours)}}.$$

The quotient is calculated to within 0·5.

The individual experiments which will now be considered are divided into three groups. In Exps. 1 to 3 (Table I) the influence of sodium and potassium

[1] Here as fresh yeast.

Table I.

Exp. no.	Date (1932)	Temperature	Duration (mins.)	Q_{CO_2}			Time interval (mins.)
					Addition of		
				Control	NaCl	KCl	
1.	9. v	25°	40	32·5*	46·5	84·5*	20–40
2.	29. vi	21°	75	31·0	58·5	75·0	30–75
3.	11. vii	21°	135	23·5	43·5	55·0	75–135

* Mean of two parallel estimations.

chlorides, acting for varying time periods, was examined. The fermentation intensity tended to alter during the observation time. With the exception of one control estimation of Exp. 1, in which there was a small decrease, this change consisted of a more or less distinct increase, apparently up to constant values. For comparison we will therefore consider, where possible, only those time intervals in which the fermentation intensity in all parts of the experiment was practically constant. It is to be noted that, compared with the figures of the corresponding control estimations, the addition of sodium chloride to the medium gives a definite increase of the fermentation intensity, while the addition of potassium chloride gives a still higher increase.

In Exps. 4 to 8 (Table II), again for varying time periods, the actions of sodium, potassium, rubidium and caesium chlorides were compared. Here the fermentation intensity as a rule increased during the observation time, but it appeared that the addition of potassium or rubidium chloride tended to bring it more rapidly nearer to a constant value. In one of the control estimations of Exp. 4 only did the fermentation intensity decrease until it gradually approximated to the value of the other control. For comparison those periods in which the fermentation intensity was not sufficiently constant in all parts of the experiment are again excluded. We see that all four alkali chlorides increase the fermentation intensity. Potassium and rubidium chlorides have always the same effect, and that of sodium chloride is always weaker than either. The position of caesium chloride is, on the other hand, variable. While in three experiments its influence is as strong, or nearly as strong, as that of potassium and rubidium chlorides, in two experiments it is weaker than that of sodium chloride. In one of these the increase obtained is near, if not within, the experimental error.

Table II.

Exp. no.	Date (1932)	Temperature	Duration (mins.)	Q_{CO_2}					Time interval (mins.)
					Addition of				
				Control	NaCl	KCl	RbCl	CsCl	
4.	28. v	25°	90	28·5*	61·0	79·0*	83·5	33·5	60–90
5.	8. vi	21°	120	12·0	28·5	35·0	36·0	36·0	60–120
6.	9. vi	21°	240	28·0	48·0	60·0	62·5	57·5	195–240
7.	20. vi	21°	180	15·0	48·0	79·0	73·5	32·5	135–180
8.	18. vii	24°	120	35·0	56·5	69·5	73·0	66·0	60–120

* Mean of two parallel estimations.

Lastly in Exps. 9 to 11 (Table III) we investigated the influence of lithium chloride, comparing it with that of potassium chloride. In Exps. 9 and 10 we also again investigated the effect of caesium chloride and in 11 that of sodium chloride. In Exp. 11 we tested in addition the effect of ammonium chloride. It may be noted that there was a striking decrease of the fermentation intensity in one control estimation of Exp. 9. Gradually the values became comparable with those of the other control in which there was only a slight decrease. Otherwise the fermentation intensity tended to increase or, notably in the tests with potassium chloride, to remain fairly constant. For comparison we have again selected only the time intervals above specified. As we see, the action of lithium chloride is in two experiments within the experimental error. The same can be said as regards ammonium chloride. In the third experiment with lithium chloride the effect is larger, due to the low value of the corresponding control; nevertheless the position of lithium chloride relatively to potassium chloride remains unaltered. Caesium chloride acts similarly to, or a little more strongly than, the latter. Sodium chloride stands in Exp. 11 approximately between lithium and potassium chloride. This is also shown especially clearly in Fig. 1, in which the whole course of fermentation in the individual tests of this experiment (with the exception of that with ammonium chloride) is given.

Table III.

				Q_{CO_2}						
					Addition of					
Exp. no.	Date (1932)	Temperature	Duration (mins.)	Control	NH_4Cl	LiCl	NaCl	KCl	CsCl	Time interval (mins.)
9.	2. vi	21°	195	13·5*	—	23·5	—	42·0	43·5	135–195
10.	3. vi	21°	165	28·0*	—	29·0	—	47·5	52·5	90–165
11.	13. vi	21°	135	26·0	29·0	28·0	42·5	65·5	—	75–135

* Mean of two parallel estimations.

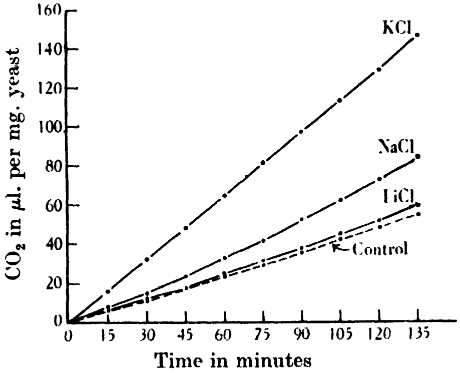

Fig. 1. Influence of 0·01 *M* lithium, sodium and potassium chlorides on fermentation of baker's yeast at 21°.

The observation that the fermentation intensity tended to increase during the course of experiments, together with the fact that in media containing potassium or rubidium chloride it was either fairly constant or showed a comparatively slight rise, indicates that when not completely activated the induction period of the fermentation was delayed, the time utilised in preparing the experiment having been insufficient to enable the fermentation intensity to reach a constant value. On the other hand the fermentation decrease, sometimes observed in the control, requires a different explanation.

The mean increase in fermentation intensity by the addition of potassium chloride, the effect of which was tested in eleven experiments, was about 150 %. In order to find the mean position in which the other alkali chlorides stand in relation to potassium chloride we cannot compare the mean values one with another, in view of the very unequal number of estimations made. For this purpose we have chosen rather the "relative mean increase" which we may define as

$$\frac{\text{mean increase by alkali chloride}}{\text{mean increase in the corresponding tests with potassium chloride}} \times 150.$$

We, in this way, obtain the following figures:

Relative mean increase in fermentation intensity by

LiCl	NaCl	RbCl	CsCl
about 20 %	about 80 %	about 150 %	about 100 %

While rubidium chloride acts as strongly as potassium chloride the mean action of sodium and caesium chlorides is only about one-half to two-thirds as large. Lithium chloride acts very feebly, if at all. It is evident that these differences must be ascribed to the influence of the cations, the chlorine ion being a common constituent. As regards the individual figures there was considerable variation in the increase of fermentation by the addition of each alkali chloride, but meanwhile we are unable to give a definite explanation for this. However large the variation may be, it will be noted that there is in each individual experiment a similar order of action of the alkali chlorides as shown by the relative mean values. The only exception to this rule is caesium chloride.

DISCUSSION.

The object of our researches was to show whether alkali cations can augment the fermentation capacity of yeast and whether there is a parallelism between the influence on fermentation and that on growth. We have seen that our results confirmed our anticipations. The potassium ion greatly increases fermentation. Rubidium acts similarly, while lithium, on the contrary, either only increases it a little or not at all. Sodium and caesium have intermediate effects. Thus it is found that the order of action is similar both for growth and fermentation, and this supports strongly the theory that increase of growth occurs as a result of an acceleration of energy metabolism. Complementary researches on the influence upon respiration appear therefore to be necessary. One must be guarded, however, about drawing conclusions as regards caesium. Its mean effect on fermentation is slightly stronger than that of sodium while the relation is rather the reverse as regards the amount of growth activation. On the other hand caesium was the only alkali cation of which the relations to potassium were not similar in all fermentation experiments. Further investigations are needed in order to explain the irregular action of caesium.

Among the five alkali cations tested potassium is probably the only one which is of importance for the life of the yeast cell under normal conditions, the sodium content of yeast being apparently very small. Potassium may thus be considered as a natural activator of yeast fermentation (and very likely of the whole energy metabolism). In view of this fact the very important question arises as to whether the observed activation of fermentation is in effect the result of the addition of different alkali cations to the potassium originally present in the yeast cell, or whether the original potassium content was lessened by our preliminary manipulation, and the subsequent addition of alkali cations compensated in a varying degree for this deficit. A satisfactory answer to this question can only be given by making ash analyses. Meanwhile we may be permitted to draw attention to the following point. The ordinary method of preparing fermentation experiments consists in washing the yeast with distilled or tap water in order to free it from adhering substances. On electrostatic grounds, under this condition a large outward diffusion of potassium is only possible if the surface of the yeast cell is permeable to cations and anions to a similar degree. That this is not so, and that the potassium content of yeast is therefore only slightly or not at all lessened has been shown by Genaud [1929]. He found that the potassium and likewise the chlorine content of wine and baker's yeast was hardly changed by washing with distilled water. If the yeast cells were, however, washed in 0·01 *M* ammonium chloride solution (with a p_H of 7·0) potassium and calcium ions came out of the yeast into the medium from which an approximately equivalent quantity of ammonium ions disappeared. The chlorine content of the yeast here also remained approximately constant.[1] Thus it is probable that the surface of the yeast cell is permeable with ease to cations but with difficulty to anions. The outward diffusion of cations must therefore be more rapid in an electrolytic medium than in the practically electrolyte-free water, for in the former an interchange of the cations takes place. But, in our investigations the yeast was washed with a relatively large amount of suspension fluid, rich in ammonium and hydrogen ions. The condition for a more rapid outward diffusion of potassium was thus present, and it is probably for this reason that we obtained, by the subsequent addition of alkali cations, a significantly greater increase of fermentation intensity than had Boas who, before commencing his experiments, only washed his yeast in distilled water.[2] Meanwhile it is questionable whether, with a single and short washing as carried out by us, there is a very significant diminution in the potassium content of the yeast. It is important that this point should be investigated, and especially also to find out whether any fermentation at all is possible in the complete or almost complete absence of potassium.

When we consider that the potassium content of yeast cells suspended in water is relatively high and that it can only be diminished by the addition of another kind of cation we must assume that the inhibitory effect of lithium, which Lindahl observed in his researches, was indirect. It may be that a certain part of the intracellular potassium is replaced by the almost inert lithium. It remains for further researches to show whether this conception is correct, and whether in addition to the indirect action there may not be a direct inhibiting influence of lithium, at least when in a high concentration.

If, as appears probable, potassium is a natural activator of alcoholic fermentation it will be interesting to try and find out at what stage of the glucose disintegration the potassium works. This can be done by the investigation of partial reactions; lines formerly worked along by Harden, and recently also by Lindahl.

APPENDIX: THE INFLUENCE OF MAGNESIUM.

We also investigated the influence of magnesium in three further experiments, using methods similar to those already described. The suspension fluid contained, as in the corresponding growth experiments, 0·015 *M* potassium chloride, and was free from magnesium. Also, as before, magnesium was added as magnesium sulphate and magnesium chloride. Further parallel estimations were made with the addition of sodium sulphate in order to test the influence of the SO_4 ions. The concentration of the addition solutions was 0·06 *M*. After mixing with the yeast suspension in the proportion of 2:1 the concentration of the added salts was therefore 0·02 *M*, 2½ times as high as the concentration of magnesium sulphate in the former experiments. The concentration of potassium chloride was as before 0·01 *M*. In two experiments each estimation was duplicated.

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[1] Similar results were obtained with magnesium by Lohmann [1931] and by Euler and his collaborators [1931] during the course of their experiments on the activation of alcoholic fermentation by magnesium. These authors, however, worked with dried yeast.

[2] However, in comparing our results with those of Boas we must also remember the differences in the conditions of the media during the fermentation experiments themselves, especially the higher concentration of the salts added by him.

Of the three experiments, the duration of which was between 60 and 120 min., two showed no influence of magnesium. In the third there was an increase in fermentation intensity of about 30 % for both magnesium salts, while sodium sulphate was without influence. Thus it appears that in the given conditions magnesium exerts little or no action on fermentation.

According to the researches of Lohmann [1931] and Euler *et al.* [1931] magnesium is an activator of alcoholic fermentation as well as of lactic acid formation in the animal tissue. It represents the inorganic part of the "co-enzyme system". Thus we cannot conclude from our result that the magnesium ion is without essential importance for the fermentation capacity of yeast. The best explanation for the indifference of magnesium in our experiments would appear to be that the intracellular magnesium was not sufficiently washed away by our technique, so that even in the control medium the activating amount of magnesium was at a maximum. The fact that magnesium ions, in consequence of their lower mobility, diffuse more slowly out of the cells than do potassium ions may account for this. On the other hand there is in our investigations the remarkable difference between the lack of dependence of the fermentation on the presence of magnesium and the activating effect of the latter in the growth experiments made under similar conditions as to media. Perhaps this difference can be ascribed chiefly to a subsequent outward diffusion of a large amount of magnesium, due to the longer duration of the growth experiments, in spite of the fact that otherwise the conditions for diffusion were less favourable.

SUMMARY.

In the present investigations we have tested the influence of alkali cations on the alcoholic fermentation of baker's yeast. The fermentation was carried out under anaerobic conditions. Our results show that the alkali cations on the whole facilitate fermentation. Individually, however, their actions are very different, as was the case in the growth experiments previously described. For instance, with potassium chloride there is a mean increase of about 150 %. Rubidium acts as strongly as potassium. On the contrary the effect of lithium is slight or zero. Sodium stands between lithium and potassium while the position of caesium is somewhat doubtful. Although in five experiments its influence was about as strong as that of potassium, in two experiments it was perceptibly weaker than that of sodium. If one leaves caesium out of account for the moment, the results of both series of investigations show a clear parallelism between growth and fermentation activation. This parallelism indicates that the activation of growth is to be attributed to an increase of the fermentation capacity (or very likely of the whole energy metabolism) of the yeast cell. In some experiments (with the addition of potassium to the medium) the influence of magnesium was also tested. Its action proved to be very slight or zero. This is explained on the grounds that the intracellular magnesium was not sufficiently washed away during the preparation of the yeast.

REFERENCES.

Boas (1921). *Biochem. Z.* **117**, 166.
Euler, Nilsson and Auhagen (1931). *Z. physiol. Chem.* **200**, 1.
Genaud (1929). *Compt. Rend. Acad. Sci.* **188**, 1513.
Harden (1917). *Biochem. J.* **11**, 64.
—— and Henley (1921). *Biochem. J.* **15**, 175.
Lasnitzki and Szörényi (1934). *Biochem. J.* **28**, 1678.
Lindahl (1933)[1]. *Arch. Entwicklungsmech.* **128**, 661.
—— (1934)[1]. *Naturwiss.* **22**, 105.
Lohmann (1931). *Biochem. Z.* **237**, 445; **241**, 50, 67.
Meyerhof (1918). *Z. physiol. Chem.* **102**, 185.
Speakman, Gee and Luck (1928). *J. Bacteriol.* **15**, 319.
Warburg (1926). Über den Stoffwechsel der Tumoren. (J. Springer, Berlin.)

[1] Supplemented by a personal communication.

LXIX. STUDIES IN THE METABOLISM OF PROTOZOA.

I. THE NITROGENOUS METABOLISM AND RESPIRATION OF *BODO CAUDATUS*.

By NORMAN RISBOROUGH LAWRIE[1].

From the Sir William Dunn Institute of Biochemistry, Cambridge, and the Bonnett Memorial Laboratory, Addenbrooke's Hospital, Cambridge.

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I. NITROGENOUS METABOLISM.

The nitrogenous metabolism of the protozoa has hitherto been much more extensively studied from the anabolic than from the catabolic aspect. This is probably due to the fact that information regarding nitrogenous food requirements is likely to facilitate culture of the protozoa, whilst the search for metabolic end-products is greatly hampered by the complexity of the cultural media which it is usually necessary to employ. A discussion of this difficulty and of the methods used in this work to minimise its effects is given later in this paper.

For a review of the nutritional requirements of the protozoa and many original contributions to this subject the reader is referred to the monograph by Lwoff [1932] and to the review of the rôle of bacteria in the nutrition of protozoa by Luck *et al.* [1931].

Griffiths [1888] stated that *Amoeba sphaerococcus*, *Vorticella* sp. and *Paramoecium bursaria* contained uric acid in their contractile vacuoles. His evidence consisted of positive murexide tests obtained upon the organisms in bulk and a flaky precipitate observable in their contractile vacuoles when the organisms were treated with alcohol. Adolph [1922] concluded that *Phagocata* and *Paramoecium* did not produce urea. Howland [1924] repeated the tests used by Griffiths with the organisms *Centropyxis* and *Amoeba verrucosa* and obtained negative results. Using the very sensitive Benedict's uric acid reagent, however, he obtained a positive reaction for uric acid in cultures of *Paramoecium* and *Amoeba*.

Weatherby [1927] concluded that *Paramoecium* produces urea, but his findings were not consistently positive using the urease test, nor was he able to demonstrate the presence of urea by the xanthydrol test. Injection of Nessler's reagent failed to show the presence of ammonia in the contractile vacuole, but the limit of sensitivity of the test under these conditions is not made clear. The same worker [Weatherby, 1929] studied the production of ammonia in suspensions of protozoa washed by decantation and suspended in salt solution. His results are summarised as follows:

Organism	Ammonia	Urea	Uric acid	Creatine	Creatinine
Paramoecium	0	+	0	0	0
Spirostomum	0	+	0	0	0
Didinium	+	0	?	0	0

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[1] Beit Memorial Research Fellow.

He found that many culture media gave a positive test for uric acid with Benedict's reagent. In the case of the *Didinium* cultures the production of ammonia was shown to vary with the food supplied to the organism.

EXPERIMENTAL.

The types of cultures of protozoa available for metabolic studies may be considered under the following three headings:

Type A. Double cultures, containing one species of protozoan and one or more species of bacterium, both protozoa and bacteria growing and multiplying actively in a medium nutrient for the bacteria.

Type B. Cultures containing one species of protozoan feeding upon live bacteria suspended in some non-nutrient medium in which the bacterial growth and multiplication is minimum.

Type C. Bacteriologically sterile cultures of protozoa.

A culture of type A would give a large metabolic turnover corresponding to very active growth and division, but on account of the extreme difficulty of separating bacterial from protozoal effects in such a culture it was not considered advisable to attempt any metabolic studies upon a culture of this type.

A culture of type B represents a compromise between types A and C. The cultures of *Bodo* used in the experiments described below belong to this type. They have the advantage of providing high rates of metabolic activity, of growth and of division, whilst the effects due to the bacteria alone can be determined with sufficient accuracy for many studies.

A culture of type C is by far the most satisfactory for metabolic studies provided sufficiently active growth and division can be obtained. Metabolic studies of cultures of this type will be presented in future communications. In the case of a very few protozoa growth takes place upon killed bacteria, and such cultures belong to this type C.

The culture of Bodo caudatus.

The culture of *Bodo caudatus* was carried out by methods almost identical with those used by Robertson [1932]. The method may be outlined as follows.

The food supply consists of a suspension of bacteria in either sterile distilled water or a simple inorganic salt solution according to the particular experiment in hand. The salt solutions were sterilised by autoclaving at 110° for 20 minutes.

The bacterium was the predominant organism in the primary cultures which were isolated from a macerating tank. It is Gram-negative, asporogenous, motile, and rod-shaped, slightly pleomorphic and produces a slight yellow colour when grown on agar slopes. The bacterium is almost inert towards the sugars and slightly proteolytic. It has been repeatedly purified by replating.

The density of the bacterial suspension is so chosen that the final concentration of bacteria in the inoculated culture shall give an opacity matching the "opacity 3" of the "Wellcome" standard opacity tubes.

This food supply is then inoculated with a measured quantity of a previous culture, the size of the inoculum being so chosen that the new culture starts with approximately 300 bodos per 1 μl. Measured samples, usually 6 ml., of cultures prepared in this way are transferred to sterile Petri dishes and kept in an incubator at 25°.

In order to estimate the number of bodos in a culture dish the contents are transferred to a test-tube and thoroughly mixed. 0·5 ml. is then transferred into 2 ml. of a saturated solution of mercuric chloride in 1·5 % aqueous acetic acid. This fixed and diluted suspension is then counted in Thoma-Zeiss or Fuchs-Rosenthal counting chambers in the usual way. Before all volumetric measurements involved in the above procedure the suspension of organisms is very thoroughly mixed by means of Pasteur pipettes.

All glassware which is used in the manipulation of these cultures is sterilised by baking at 120° for 30 minutes.

BIOCHEMICAL FINDINGS.

The total nitrogen present in a culture prepared as above with a bacterial suspension of "opacity 3" density was found to be approximately 10 mg. of ammonia-nitrogen per 100 ml. of culture. In order therefore to study fluctuations in a fraction of this total nitrogen it was essential to use a delicate method of estimation. This consideration largely determined the use of the following methods.

Estimation of the ammonia content of the cultures.

The culture was first deproteinised with "colloidal iron" (B.D.H.). To 4 ml. of the culture in a 15 ml. centrifuge-tube was added 0·3 ml. of "colloidal iron." After mixing the contents of the tube thoroughly one drop of a saturated solution of potassium sulphate was added to complete the precipitation of the iron. The preparation could now stand for 24 hours if necessary without alteration of its ammonia content. Otherwise, the preparation was centrifuged at once, for 10 minutes at 3000 r.p.m. Alternatively, the precipitate could be filtered off. 2 ml. of the perfectly clear supernatant fluid were then transferred to a test-tube, and 5 ml. of a 0·1 % solution of gum ghatti in water saturated with thymol was added. After mixing by inversion, 1 ml. of Nessler's reagent was added. A series of standards was simultaneously prepared and nesslerised. The tubes were matched, after standing for 20 minutes, in a Klett-Bio or Bausch and Lomb colorimeter, using a heavy blue screen over the eyepiece. The value for the ammonia-nitrogen per 100 ml. of culture thus obtained was multiplied by 1·08 to correct for the dilution with "colloidal iron." Duplicate estimations carried out by this method agreed within 5 % and usually within 3 %. Recovery of ammonia added as ammonium sulphate was satisfactory as the following example shows:

Bacterial suspension washed free from ammonia and ammonium sulphate added to give a concentration of	2·00 mg. ammonia-N/100 ml.
Ammonia found by estimation	1·97 ,,
Loss	1·5 %

Total nitrogen was estimated by a micro-Kjeldahl incineration and aeration of the ammonia formed into standard acid. This receiving acid was then neutralised and nesslerised. Standard ammonium sulphate solutions were aerated in exactly the same way.

Nitrogen partition in the cultures.

It was necessary at the outset of these experiments to determine to what extent ammonia formation accounted for the whole of the end-products of the nitrogenous metabolism of *Bodo*. The fraction "non-protein-nitrogen other than ammonia-nitrogen" was therefore estimated at the time of inoculation and after

24 hours' growth of two strains of *Bodo*, cultured simultaneously and fed upon the same stock of bacterial suspension. The results are tabulated below.

	Non-protein-nitrogen other than ammonia-nitrogen mg. per 100 ml. of culture	
	(*a*) At the start of the culture	(*b*) After 24 hours' growth
Bacterial suspension 58	0·65	0·36
Bodo culture N 58	0·72	0·83
Bodo culture R 56	0·82	0·62
Bacterial suspension 59	0·57	0·42
Bodo culture N 59	0·96	0·89
Bodo culture R 57	1·02	0·80
Bacterial suspension 60	0·49	0·37
Bodo culture N 60	0·68	0·58
Bodo culture R 58	0·51	1·09

A consideration of these figures led to the conclusion that the non-protein-nitrogen other than ammonia-nitrogen was probably largely derived from the food supply and did not show variations which could be correlated with the growth and multiplication of *Bodo*.

No urea could be demonstrated in the deproteinised filtrates from cultures by the xanthydrol test or by treatment with urease preparations. No uric acid could be demonstrated in these filtrates by means of Benedict's uric acid reagent [Benedict and Franke, 1922]. Neither urea-nitrogen nor uric acid-nitrogen can then be present in these filtrates to a greater extent than 3 % of the total non-protein-nitrogen formed. The cultures showed no urease activity.

Production of ammonia during the development of the cultures.

In order to follow the production of ammonia throughout the life of the culture a stock of bacterial suspension of standard opacity 3 containing about 300 bodos per μl. was prepared in a large boiling-tube. After thorough mixing, this culture was distributed throughout a series of Petri dishes, 6 ml. of culture being measured into each dish. The cultures were incubated at 25° and representative dishes removed at appropriate intervals for counting of bodos and estimation of ammonia.

The bacterial suspension itself produces ammonia at a slow and approximately constant rate. It was only considered necessary therefore to measure the ammonia contents of the controls in these experiments at the start and end of the period of observation.

The relation of ammonia production to the development of the cultures was then studied by means of a series of daily subcultures carried out under conditions as nearly constant as practicable. Thus the transfer was so calculated each day as to produce a population of 300 bodos per μl. in the new culture. Each culture was allowed to grow for the same length of time, 22 hours, before being subcultured. The handling of the cultures and exposure to room temperature which this involved were kept as constant as possible, and 22 hours of each culture's life were spent in the incubator at 25°. The bacterial food supply was prepared freshly each day from 24-hour old cultures on agar slopes, and it contained approximately 10 mg. of total nitrogen per 100 ml. After a few days' subculture under these conditions the cultures settled down to a steady rate of development, so that the extent to which they had to be diluted in making a new subculture tended to a constant figure (about × 20). Several Petri dishes were then set up from a common stock and dishes withdrawn for counting of bodos and estimation of ammonia at suitable intervals. The results of four such successive subcultures are shown in Fig. 1, in which the ammonia production of cultures and controls is shown. The dilution figures used in preparing cultures N 50 to N 53 were 23, 21, 20 and 23 respectively. These figures are indicative of the degree of reproducibility of the rate of multiplication of the bodos.

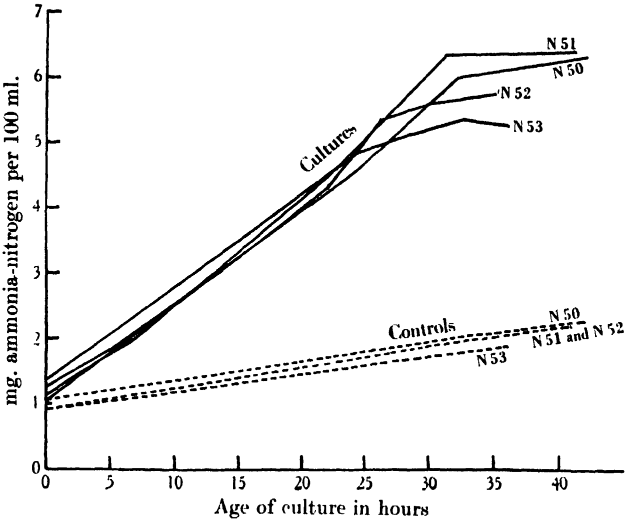

Fig. 1.

The following are the main features brought out by this figure:

(*a*) The contribution of the bacterial suspension alone is only a small fraction of the total ammonia produced.

(*b*) The total ammonia-nitrogen produced by the cultures less that produced by the corresponding controls was 3·9, 4·0, 3·7 and 3·0 mg. per 100 ml., *i.e.* an average of 3·7 mg. or 37 % of the total nitrogen in the culture.

(*c*) The bodos were multiplying rapidly until about the 24th hour, when their rate of multiplication fell off. About this time too encystation often appeared in the cultures. Thus the rapid formation of ammonia is associated with the period of rapid multiplication of the bodos rather than with the period just before the onset of encystation.

A similar and more complete set of curves was then obtained during experiments carried out in collaboration with Dr Robertson. Different strains of *Bodo* were used, grown, however, under conditions almost identical with those described above. The controls were obtained by a different procedure. A portion of the freshly prepared culture was set aside at 40° for a constant period of 2 hours. This treatment sufficed to kill all the bodos but left the bacteria alive. This killed bodo culture was then set up in Petri dishes in exactly the same way as the live culture. It is to be noted that every element in the culture is here represented in the control with the exception of the live bodos. This control is of course somewhat exaggerating the bacterial effect as all the bodos in the new control are here available for food supply for the bacteria, whilst the bacteria are not themselves being consumed as in the live bodo cultures.

In interpreting the figures for the ammonia contents of the cultures the ammonia gained by the control was deducted from the total ammonia present in the live culture to get a corrected curve of ammonia production for the live culture. From this corrected curve of ammonia production the rate of ammonia production was calculated for each period between two observations. Fig. 2 shows how this rate of ammonia production and the number of bodos present vary with age in the case of three representative cultures.

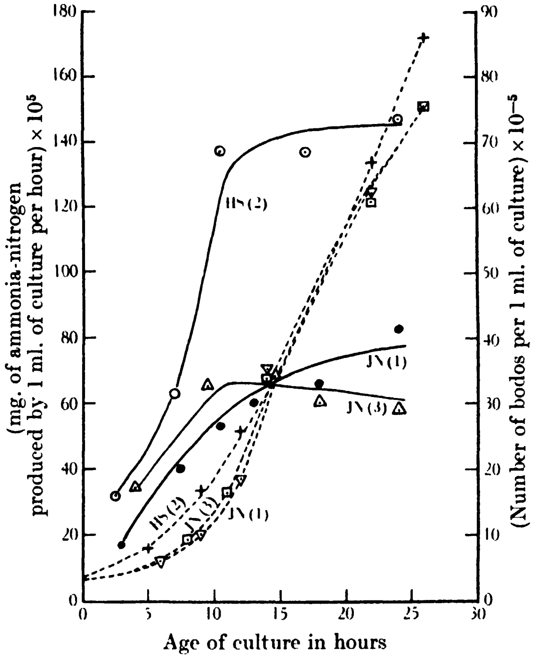

Fig. 2. Rate of ammonia production by: culture HS(2) o—o; culture JN(1) •—•; culture JN(3) ▵—▵. Number of bodos: culture HS(2) ×---×; culture JN(1) ▿---▿; culture JN(3) ▫---▫.

Considering first the curves of rates of ammonia production, it is seen that the initial rate of ammonia production is more than doubled by the time the culture is 8–10 hours old and thereafter remains steady or increases relatively slowly till the end of the period studied. It is at just this period when the rate of ammonia production ceases to increase rapidly that the bodos pass out of their period of lag in multiplication and enter upon a period of very rapid increase in numbers which is maintained to the end of the period studied. Thus from about the 8th to 10th hour onwards a roughly steady rate of ammonia production is being maintained by a rapidly increasing number of bodos, or in other words the rate of ammonia production per bodo falls steadily and rapidly from the 8th to 10th hours onwards until about the 26th hour of growth.

In the case of measurements of ammonia production made during the early hours of the culture's development the figures are small differences between relatively large quantities, and it would not therefore be justifiable to attempt to draw any more precise conclusion than that during this period the rate of ammonia production is very markedly low in comparison with the steady rate which is established later.

The possible significance of this feature of nitrogenous metabolism will be discussed at the end of this paper in relation with curves of oxygen uptake and certain morphological data.

II. RESPIRATION.

A number of more or less quantitative experiments have been carried out to determine what oxygen tension is most favourable for the development of various protozoa. Such experiments involve observation of the migration of protozoa in cultures into oxygen-rich or oxygen-poor localities and of the influence of altered oxygen tension upon the behaviour and viability of cultures. These experiments have indicated a pronounced sensitivity of protozoa to the oxygen tension of their environment. The work of Fox [1920–21] is particularly to be noted in this connection. Fox worked with a culture of *Bodo sulcatus* obtained from 6-day old infusions of grass. Square cover glasses were mounted over microscope slides and samples of the cultures run in between the cover glasses and slides. The preparations were kept in moist chambers and periodically examined under the microscope. At first the bodos were uniformly distributed, but after a time, varying from a few minutes to a day, they collected in the centre of the cell. Subsequently an area free from bodos appeared in the middle of the clump, and the ring of bodos thus formed expanded till it took up a position at a uniform distance from the edge of the cover slip. Finally these migrations were reversed and the bodos again clumped in the centre of the cell. The influence of bubbles of air and other gases, of solid particles, of de-oxygenating reagents and increased oxygen tension upon their behaviour was studied. The conclusion was drawn that *B. sulcatus* is very sensitive to changes in the oxygen tension around the optimum oxygen tension for its development and migrates to the region of optimum oxygen tension.

In connection with experiments to be described in this paper it is of particular interest to note that Fox has occasion to refer to the possibility of a change in the optimum oxygen tension occurring during development of the culture. Such a change might explain the migration of the bodos from the edge of the cell to the centre on allowing the cultures to stand in the moist chamber, and also, to a certain degree, the shape of the ring of bodos as viewed in vertical section.

Soule [1924] studied the respiration of *Trypanosoma lewisi* and *Leishmania tropica* grown on a blood-broth-agar medium. When his cultures were supplied with 100 ml. of air *Leishmania* used all the oxygen in 144 hours, whilst the trypanosomes required twice that time to consume all the oxygen. When the cultures were supplied with 2 litres of air the rates of oxygen consumption and carbon dioxide output were approximately equal for both species of protozoa. Excess of oxygen or anaerobiosis inhibited growth. Carbon dioxide was toxic in concentrations of over 20 %. The respiratory quotient for *Leishmania* measured from the time of inoculation to the seventh day of growth was 0·84, and that for the trypanosomes 0·74. It was suggested that much base was produced to account for the carbon dioxide retained in the culture. No counts were made. Degeneration was always present at the end of the experiments.

Fenyvessy and Reiner [1924] showed that cultures of trypanosomes gave a vigorous oxygen uptake and carbon dioxide output when studied by means of the Barcroft differential manometer. Their work was continued [1928] with the Warburg respirometer, and a washed suspension of trypanosomes in Ringer's solution containing glucose was used. The gaseous exchange was observed for periods of about 20 minutes. The glucose was decomposed by both oxidation and "glycolysis"; the latter being greatest during anaerobiosis. The very interesting observation was made that neither germanin nor potassium cyanide inhibited either process when added to the suspension in any concentration up to 0·1 %. This finding is the more remarkable in that germanin is toxic to trypanosomes in their vertebrate host. If the p_H of the system is lowered "glycolysis" is increased at the expense of oxidation and *vice versa*. These workers found aerobic and anaerobic "glycolysis" to proceed at the same rate. The rate of oxygen uptake per 10^8 organisms per hour varied from 3·7 to 7·5 μl., and it is very interesting to note that in experiments to be described immediately the same number of the very much smaller organism *Bodo caudatus* consumed 350 μl. of oxygen in the same time. In the case of the trypanosomes the organisms were in a medium which was non-nutrient for them, whilst the bodos were metabolising foodstuffs, growing and multiplying very actively.

EXPERIMENTAL.

The oxygen uptake of cultures of *B. caudatus* was measured by means of the Barcroft differential manometer. The cultures were prepared in bulk in boiling-tubes as described earlier in this paper. Control material was prepared by heating a portion of these newly inoculated cultures to 40° for 2 hours, which process kills the bodos but not the bacteria. 5 ml. of live culture were measured

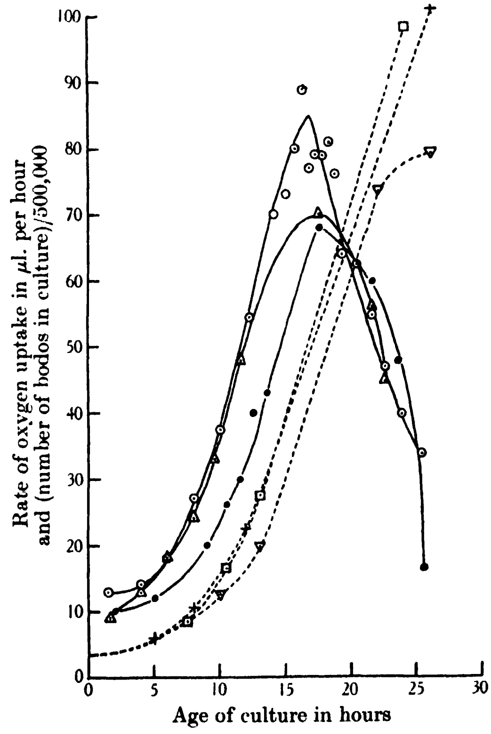

Fig. 3. Rate of oxygen uptake by: culture JN(1) •—•; culture JN(3) △—△; culture JN(7) ○—○; Number of bodos: culture JN(1) ▽---▽; culture JN(3) □---□; culture JN(7) ×---×.

into the right-hand cups of four differential manometers and 5 ml. of killed bodo control material into the left-hand cups. The water-bath was maintained at a temperature of 25°. At intervals throughout the next 24 hours cups were removed, the contents analysed for ammonia and the numbers of bodos per μl. counted. The manometer cups were cleaned particularly scrupulously but were not sterilised, since the bodo culture is not likely to provide a medium which would allow of significant bacterial growth within the period of these experiments. The oxygen uptake of the control was of course automatically allowed for by the differential character of the apparatus. The results of three experiments of this type are shown graphically in Fig. 3. In this figure the rate of oxygen uptake per hour for the whole culture and the number of bodos in the culture are plotted against the age of the culture in hours.

It is seen that a most pronounced change occurs in the relationship between the rate of oxygen uptake and numbers of bodos present about the 16th to 18th hours. Up to this time the two curves have run almost parallel, implying that the rate of oxygen uptake is directly proportional to the number of bodos present throughout this period. Between the 16th and 18th hours the rate of oxygen uptake reaches a sharp peak value and then starts to fall rapidly. This occurs despite the fact that the number of bodos in the culture is still increasing. After the 16th to 18th hour, however, the generation time is always increasing; its minimum value having occurred between the 8th and 16th hours of development of the culture. By the 22nd to 24th hours the rate of oxygen uptake has fallen to about one-half of its peak value, although there are then nearly twice as many bodos in the culture as there were at the time of most rapid oxygen uptake. Very good confirmation of the earlier parts of these curves, up to the peak value, was obtained in the study of another strain of *Bodo*. With this strain, however, the experiments were not carried on sufficiently long to follow the curve after the 18th hour.

Variations in size of Bodo caudatus.

It is to be noted that the size of the bodos varies greatly at different ages of the culture, so that the curves of the numbers present give only a limited picture of the morphological development of the culture. Dr Robertson has kindly placed at my disposal a large series of unpublished measurements of individual bodos at various ages of the cultures. In Fig. 4 the data giving the area distribution curves for the typical cultures HN (2) and HN (13) are summarised. In these curves a decrease in the proportion of large bodos present is indicated by a flattening of the curve, and a decrease in the average size of the bodos present is indicated by a shift of the curve as a whole towards the left. As a matter of convenience area × 4 is plotted instead of the actual area. These measurements were obtained by tracing the outline of bodos by means of the camera lucida. Hence the area measured is that projected by the bodo upon the plane surface upon which it is lying and not the actual surface area. It is clear from this figure that the proportion of small bodos increases very greatly between the 11th or 12th and the 22nd hours. Thus there are from three to four times as many bodos of 50 units size at 22 hours' growth as there were at any previously measured period. Moreover, there are hardly any bodos bigger than 100 units at 22 hours although at previously measured periods such large individuals were numerous.

It is during this period when multiplication is outpacing individual growth that the marked peak in the rate of oxygen uptake by the culture occurs (Fig. 3). It must be borne in mind that during this period (the 12th to 22nd hours) no dead bodos are appearing in the cultures, encystation is not occurring and motility does not appear to be in the least diminished. It would appear then that during this period some form of anaerobiosis is supplying the energy requirements of the organisms to an increasing extent. The occurrence of this biochemical change during a period of pronounced morphological change must be noted, although any correlation between these two types of change is not at present apparent. If anaerobiosis did indeed play an important part in the metabolism of cultures of *B. caudatus* during their later phases, it would be reasonable to expect their optimum oxygen tension to be lower in these later phases. The work of Fox [1920–21], quoted above, may indicate such an effect in the case of *B. sulcatus*, although it must be emphasised that Fox was able to account for his results without assuming any change in the optimum oxygen tension for *B. sulcatus*.

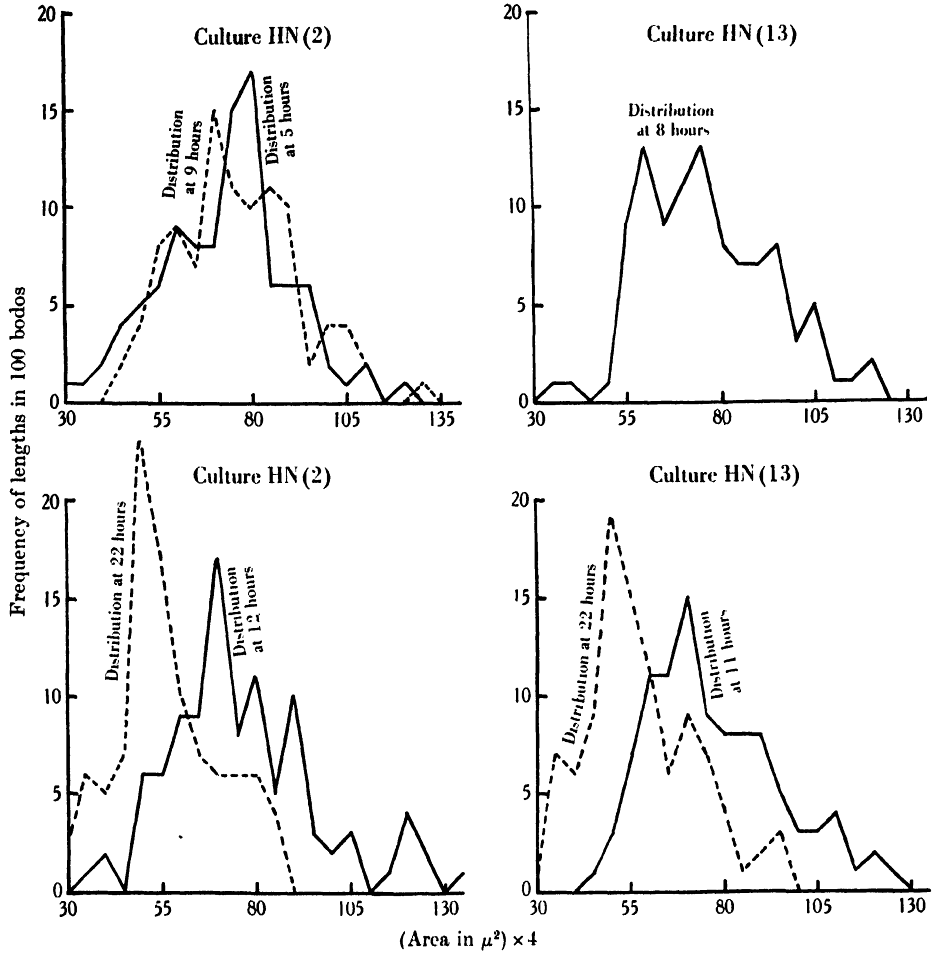

Fig. 4.

The development of the culture may be divided into two phases with respect to the three aspects for which data are here presented. In the case of nitrogenous metabolism, as studied by ammonia production, there is an early period of relatively great activity, when the rate of ammonia production runs roughly parallel with the increase in the numbers of bodos in the culture. This period lasts till about the 8th hour of growth. The second phase then commences, and during this period the rapid increase in numbers of bodos is no longer paralleled by an increase in the rate of ammonia production, which becomes steady or shows only relatively slow increase. The motility of the organisms is maintained during this second phase and no death or encystation is seen. A probable inference from these observations would be that during the early development of the culture, taking place in the presence of abundant food supply and in a low concentration of growth products, nitrogenous foodstuffs are preferentially metabolised, whilst during the later life of the culture, when metabolic products are accumulating and food supplies decreasing, the food requirements of the organism are met by the preferential metabolism of non-nitrogenous food materials.

The two phases brought out by the curve of rate of oxygen uptake have already been discussed and their time relationship to the morphological changes noted.

SUMMARY.

Data are presented for the production of ammonia, consumption of oxygen, rate of multiplication and growth of the protozoan *Bodo caudatus*. The metabolic significance of these findings is discussed.

I wish to express my thanks to Sir F. G. Hopkins for his interest in this work and to Dr M. Robertson both for her generous advice and hospitality at the Lister Institute of Preventive Medicine and for providing me with the strains of *Bodo caudatus* used in this work. I have also to thank Dr Robertson for permission to quote certain of her unpublished data.

REFERENCES.

Adolph (1922). *Anat. Rec.* **24**, 396.
Benedict and Franke (1922). *J. Biol. Chem.* **52**, 387.
Fenyvessy and Reiner (1924). *Z. Hyg. Infekt.* **102**, 109.
—— —— (1928). *Biochem. Z.* **202**, 75.
Fox (1920–21). *J. Gen. Physiol.* **3**, 483.
Griffiths (1888). *Proc. Roy. Soc. Edinburgh*, **16**, 131.
Howland (1924). *J. Exper. Zool.* **40**, 231.
Luck, Sheets and Thomas (1931). *Quart. Rev. Biol.* **6**, 46.
Lwoff (1932). Recherches biochemiques sur la nutrition des protozoaires. (Masson et Cie. Paris.)
Robertson (1932). *Quart. J. Micr. Sci.* **75**, 511.
Soule (1924). *J. Infect. Dis.* **36**, 245.
Weatherby (1927). *Biol. Bull.* **52**, 208.
—— (1929). *Physiol. Zool.* **2**, 375.

LXX. THE COMPONENT GLYCERIDES OF HEN BODY FATS.

By THOMAS PERCY HILDITCH
and WILLIAM JOHN STAINSBY.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received January 18th, 1935.*)

The methods which were recently employed in the study of the glyceride structure of a pig depôt fat [Hilditch and Stainsby, 1935] have been applied to hen body fats, the component fatty acids of which were discussed by Hilditch *et al.* [1934]. For this purpose the material (abdominal and gizzard fats) which had been left over from the last-mentioned work was utilised, since the full analysis of the total component fatty acids of a fat is essential for the study of its component glycerides. The quantity of fat thus remaining at our disposal was not very large, but by using both abdominal and gizzard fats (the component acids of which (*cf.* Table I) were almost identical) it proved possible:

(i) to ascertain the proportion and nature of fully-saturated glycerides present in the original fat;

(ii) to carry out fractional crystallisation of a specimen of completely hydrogenated hen body fat;

(iii) to make a complete analysis of a specimen of the fat after it had been hydrogenated to a point (iodine value 14·6) at which (by analogy with pig depôt fat) it was expected that all dipalmito-glycerides would have passed into the fully-saturated portion; and to carry out fractional crystallisation of the fully-saturated glycerides present in this partially hydrogenated fat.

The results obtained show that, although the fatty acids present in hen body fats are, in many respects, not dissimilar from those of an animal such as the pig, the composition of the mixed glycerides is quite different. The glycerides are much more heterogeneous in character: tri-C_{18}-glycerides and dipalmito-mono-C_{18}-glycerides are present in much greater quantities than in pig depôt fat, with a corresponding diminution in the amount of monopalmito-di-C_{18}-glycerides.

The glyceride structure of bird fats is referred to by Amberger and Bromig [1921] and Bömer [1922], both of whom submitted goose body fat to fractional crystallisation. Amberger and Bromig detected dipalmitostearin, oleodipalmitin, palmitodiolein and possibly triolein, whilst Bömer suggested that the fat probably contained about 45 % triolein, 30 % palmitodiolein, 5 % dioleostearin, 3–4 % of dipalmitostearin and a very small amount of palmitodistearin.

EXPERIMENTAL.

The abdominal and gizzard fats employed were from the batch of 24 Light Sussex hens, which Mr Halnan and Dr Cruickshank of the Cambridge School of Agriculture had kindly placed at our disposal. The diet of the fowls and the general characteristics of the fats were described in the earlier paper [Hilditch *et al.*, 1934, p. 787], while the component acids of the fats are, for reference, again quoted below (Table I). The chief features of the component acids include the palmitic acid content (27 %—a characteristic proportion for a land animal fat), low stearic and high linoleic acid contents and the presence of 8 % of the unsaturated palmitoleic ($\Delta^{9:10}$-hexadecenoic) acid.

Table I. *Component fatty acids of depôt fats from 24 Light Sussex hens (aged 7 months).*

	Weight percentages		Molar percentages	
Acid	Abdominal	Gizzard	Abdominal	Gizzard
Myristic	0·1	0·1	0·1	0·1
Palmitic	25·6	25·3	27·3	26·9
Stearic	7·0	7·1	6·7	6·8
Palmitoleic	7·0	7·6	7·6	8·2
Oleic	38·4	36·6	37·1	35·4
Linoleic	21·3	22·8	20·7	22·2
C_{20-22} unsaturated	0·6	0·5	0·5	0·4

Fully-saturated glycerides of the hen depôt fat.

These formed only a small proportion (2·5 %) of the whole fat. In this respect the fat is comparable with rat, rabbit or the more unsaturated varieties of pig depôt fats, which also contain only small quantities of stearic acid, in addition to the more or less constant amount (25–30 %) of palmitic acid.

The gizzard fat (189 g.) was oxidised in acetone solution with powdered potassium permanganate, the unchanged neutral products of oxidation were resubmitted to similar oxidation until they possessed a negligible iodine value. Fully-saturated glycerides (6·8 g.) were thus isolated in a somewhat impure condition (iodine value 0·8, acid value 19·0 and including 0·32 g. of unsaponifiable matter).[1] After applying a correction for the unsaponifiable matter and acidic compounds (calculated as azelaodipalmitin) present in the crude fully-saturated glycerides, the content of the latter in the fat was calculated to be 2·5 %. Their mixed fatty acids (M.P. 59°) had a mean molecular weight of 260·3, corresponding with a mixture of about 85 % palmitic and 15 % stearic acids; the glycerides, after several crystallisations from ether, melted at 60°, and evidently consisted mainly of tripalmitin with some palmitostearins.

Estimation of tri-C_{18}-glycerides in the hen depôt fat.

(*i*) *Fractional crystallisation of the completely hydrogenated abdominal fat.* After hydrogenation to as low an iodine value as possible (1·5), the fat (77·7 g.) was submitted to systematic fractional crystallisation from ether. Six crystallisations in all were performed, the volumes of ether employed varying from 10 to 30 ml./g. of glycerides crystallised. Table II shows the weights and analytical characteristics of the fractions finally obtained, given in increasing order of solubility.

Assuming that fraction A (M.P. 68°) is a mixture of tristearin (sap. equiv. 296·7) and palmitodistearin (sap. equiv. 287·3), the fat (77·7 g.) contained 21·4 g. of tristearin, corresponding to a molar content of 27 % of tri-C_{18}-glycerides in the whole fat. If all the glyceride unsaturation in fractions E, F, G is credited to oleodistearin, a further 3 % of tri-C_{18}-glycerides must be added; for reasons which will appear later however it is quite possible that, in this particular hydrogenated fat, any residual unsaturation is due as much to oleodipalmitin as to oleodistearin.

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[1] Part of the unsaponifiable matter (0·14 g.) was a yellow crystalline material, M.P. 120°, which was insoluble in ether.

Table II. *Fractional crystallisation of completely hydrogenated (abdominal) hen fat.*

(77·7 g., I.V. 1·5.)

		Glycerides		Corresponding acids (freed from unsap.)	
No.	g.	Sap. equiv.	I.V.	Sap. equiv.	I.V.
A	42·1	292·0	—	—	—
B	2·7	287·2	—	—	—
C	6·3	284·3	—	—	—
D	4·4	282·3	—	—	—
E	6·3	279·8	2·6	267·3	2·5
F	9·2	279·4	1·0	266·7	1·1
G	6·7	287·7	9·5	268·9	8·7

If fractions B to G are similarly dealt with, on the basis of the observed equivalents, as binary mixtures of palmitodistearin and dipalmitostearin, or dipalmitostearin and tripalmitin, the original fat appears to have contained about 40 % of mono-C_{16}-di-C_{18}-glycerides, 29 % of di-C_{16}-mono-C_{18}-glycerides and 1 % of tripalmitin. In presence of more than small amounts of dipalmitostearin however the quantitative separation of the latter from monopalmitodistearin and tripalmitin becomes uncertain, and it is probably better to derive the composition of these groups from the total amounts of C_{16} and C_{18} acids present in the original fat, after deducting (*a*) the C_{18} acids determined as tristearin (in fraction A) and (*b*) the palmitic acid present as tripalmitin in the fully-saturated glycerides of the original fat.

On the latter basis, the results of the fractional crystallisation of the completely hydrogenated fat indicate the following approximate molar composition of the original glycerides: tri-C_{18}, 28–29 %; mono-C_{16}-di-C_{18}, 41 %; di-C_{16}-mono-C_{18}, 28–29 %, and tripalmitin, 2 %.

(ii) *Examination of a partially hydrogenated abdominal fat* (*I.V. 14·6*). Lack of material compelled us to confine our investigation to a single partially hydrogenated fat, instead of studying a series of these fats as in our previous work on the pig depôt fats [1934]. The abdominal fat (100 g.) was hydrogenated until its iodine value was reduced to 14·6. The product (84·4 g.), after oxidation with potassium permanganate in acetone, yielded 50·7 g. of crude fully-saturated glycerides (I.V. 0·6). Subsequent purification of the latter (50·0 g.) gave the following products: (*a*) almost pure fully-saturated glycerides, 47·2 g. (acid value 0·2, sap. equiv., 285·6); (*b*) less pure fully-saturated glycerides, 1·9 g. (acid value 8·2); and (*c*) acidic products of oxidation, 0·9 g. (acid value 130·0). The content of fully-saturated glycerides was therefore 58·8 % (wt.) or 58·5 % (mol.).

Fractionation of the methyl esters (27·2 g.) from the purified glycerides (*a*) showed that the component acids present were:

	% (wt.)	% (mol.)
Palmitic	32·8	35·1
Stearic	67·2	64·9

The general structure of the partially hydrogenated fat was thus as shown in Table III. Here however it is not possible, in the present instance, to differentiate between the saturated and unsaturated acids of the respective C_{16}- and C_{18}-series, because the shortage of material also prevented a separate analysis of the component acids of the whole fat. The latter can readily be derived by calculation from the iodine value of the fat and the corresponding figures for the original fat (Table I), but, the individual amounts of palmitoleic and of oleic acids remaining unhydrogenated being undetermined, it is only possible to give the total molar contents of the C_{16} and C_{18} acids in each group. Since we are only concerned, for the moment, with the relative proportions of C_{16} and C_{18} acids in the mixed saturated-unsaturated glycerides, this information is sufficient for the purpose.

Table III. *Component acids (mols.) in the fully-saturated and non-fully-saturated parts of the hydrogenated hen fat of* I.V. *14·6.*

	Whole fat	Fully-saturated	Mixed saturated-unsaturated glycerides (by difference)
Mols.	100	58·5	41·5
Myristic	0·1	—	0·1
C_{16} (Palmitic + palmitoleic)	34·9	20·5	14·4
C_{18} (Stearic + oleic)	64·5	38·0	26·5
C_{20-22}	0·5	—	0·5

If, as in other fats which have been studied, the remaining mixed saturated-unsaturated components were at this stage only tri-C_{18}-glycerides with di-C_{18}-glycerides containing only one C_{16} (or other) radical, the 15 equivalents of non-C_{18} acids present in the mixed part would require, in order to form mono-C_{16}-di-C_{18}-glycerides, more C_{18} acids (30 equivalents) than are actually present. In other words, di-C_{16}-glycerides must still be present in quantity in the mixed saturated-unsaturated part of the hydrogenated fat at present under discussion.

This support of the evidence given by the fractional crystallisation ((i) above) of the completely hydrogenated fat received additional confirmation when the purified fully-saturated glycerides (*a*) (14·27 g.) from the partially hydrogenated fat were systematically crystallised from ether into the fractions summarised in Table IV.

Table IV. *Fractional crystallisation of purified fully-saturated glycerides (a) from the hydrogenated hen fat of* I.V. *14·6.*

		Glycerides		Corresponding acids (freed from unsap.)	
No.	g.	Sap. equiv.	I.V.	Sap. equiv.	I.V.
A	4·82	291·9	—	—	—
B	3·75	286·2	—	—	—
C	3·30	283·6	0·7	271·0	0·7
D	2·40	282·5	2·1	269·8	2·2

Estimated as in the former case (*cf.* Table II, *et seq.*) these figures show that the molar composition of these fully-saturated glycerides was approximately 16 % tristearin, 65 % palmitodistearin, 14 % dipalmitostearin and 3 % tripalmitin (with 2 % of residual unsaturated glycerides, which might be either oleodipalmitin or oleodistearin). Since these fully-saturated components amounted to 58·5 % of the hydrogenated fat of iodine value 14·6, the proportions of the various glycerides present in the latter fat as a whole were respectively about 10 % tristearin, 38 % palmitodistearin and 8·5 % dipalmitostearin (with 2 % tripalmitin).

DISCUSSION.

Although, as we have already seen, the presence of palmitoleic acid, in addition to oleic and linoleic acids, in combination in the hen depôt fats complicates the interpretation of the experimental data, the latter afford a fair amount of information with regard to the component glycerides present and also give rise to certain suggestive inferences.

The very minor amounts of fully-saturated components in the original fat are, as already mentioned, quite in keeping with what has been observed in other animal fats of similar total palmitic and stearic acid contents. The non-fully-saturated glycerides however are sharply differentiated from other animal depôt fats which we have studied, in that they contain far more tri-C_{18} (unsaturated) glycerides and especially di-C_{16}-mono-C_{18} glycerides, with proportionately less monopalmito-di-C_{18}-glycerides than in other cases.

In considering the foregoing data, we would next direct attention to the following points:

(i) *The monopalmitodistearin produced by hydrogenation of hen fat.* If we compare the proportions of the various fully-saturated derivatives present in the completely and the partially hydrogenated hen fats, it is clear that palmitodistearin has been formed from palmitodi-"oleins" far more rapidly and completely than the dipalmitostearin results from its precursors:

Table V.

Mols. present (per 100 mols. of fat) of:	Completely hydrogenated fat	Partially hydrogenated fat
Tristearin	28–29	10
Monopalmitodistearin	41	38
Dipalmitomonostearin	28–29	8·5
Tripalmitin	2	2

The relative ease with which the monopalmito-derivatives pass into palmitodistearin is exactly parallel with the behaviour observed in pig depôt and other fats, and it is reasonable to assume that the same glycerides are concerned in each case, *i.e.* hydrogenation of β-monopalmitodi-"oleins" to β-palmitodistearin. If this view be accepted, it also follows that palmitoleic acid is not present to any appreciable extent in the form of monopalmitoleo-di-C_{18}-glycerides.

(ii) *Inferences regarding the dipalmitostearins produced by hydrogenation of the hen fat.* During hydrogenation, the monopalmitodistearin is not only produced much more rapidly than dipalmitostearin and tristearin, but the (slower) rates of production of each of the two latter glycerides are closely similar (*cf.* Table V): this naturally suggests a single cause for the relatively slow conversion into fully-saturated glycerides in the cases of both dipalmitostearin and tristearin. It was mentioned in our previous communication [1935] that, pending the results of configurational studies directed towards establishing the mechanism of such preferential hydrogenation, we incline to the view, as a working hypothesis, that the observed differences may be connected with relative difficulty of hydrogenation of an oleic radical in the β-, as compared with that of one in an α-position. In the present case, on this hypothesis, it follows that the unsaturated precursors of dipalmitostearin (as well as of tristearin) will contain an unsaturated acyl radical attached to the β-carbon atom of the glycerol molecule.

If this be so, any palmitic radicals originally present as such in these di-C_{16}-glycerides must have been attached to α-glyceryl carbon atoms. There remains however a further consequence of these arguments: if the mono-C_{16}-glycerides (*v. supra*) are wholly or for the most part β-monopalmitodi-"oleins", the 8 % of palmitoleic acid present in the original fat must be assigned to the di-C_{16}-glycerides now under consideration. The latter would then, for the most part, be formulated as either α-palmito-β-oleo-α'-palmitolein or α-palmito-β-palmitoleo-α'-olein. It is of some interest that the palmitoleic acid content of the whole fat (8 % mol.) would suffice to give 24 % (mol.) of these glycerides, as compared with the observed total content (p. 601) of 28–29 % of di-C_{16}-glycerides.

It is also interesting to note that, in glycerides of this type, palmitoleic acid would be acting as an individual and independently of any other fatty acid. In other words, the glyceride structure formulated seems to preclude the view that palmitic and palmitoleic acids might be regarded as interconvertible, by means of desaturation or saturation.

(iii) *Distribution of the stearic acid (7 mols. %) present in the original fat.* Apart from the insignificant amount of stearic acid combined as palmitostearins in the 2·5 % of fully-saturated glycerides, all of this acid must be in the form of mixed saturated-unsaturated glycerides. It may be present (*a*) as α-stearodi-"olein", (*b*) as β-palmitostearo-"olein", or (*c*) possibly as α-palmito-β-palmitoleo-α'-stearin. If it were present exclusively in any one of these forms, the quantity of the respective mixed glyceride would be about 20 % (mol.) of the whole fat.

There is naturally no evidence from our experimental data as to the precise mode of combination of this stearic acid. It might be confined to one isomeride; perhaps more probably, it may be distributed amongst the different types. In the summary of component glycerides (Table VI) the three possibilities can therefore merely be indicated without offering any precise solution.

Table VI. *Approximate general composition of the glycerides of hen depôt fat.*

General type		% (mol.)
Fully-saturated	Mainly tripalmitin with small amounts of palmitostearins	2
Tri-C_{18}-glycerides	Tri-unsaturated (oleo-linoleins), but may include up to 20 % (mol.) of α-stearodi-"olein"	28–29
(β-) Monopalmitodi-C_{18}-glycerides	β-Monopalmitodi-"oleins", but may include up to 20 % (mol.) of β-monopalmitostearo-"olein"	41
Di-C_{16}-mono-C_{18}-glycerides	Probably α-palmito-"oleo"-palmitoleins, but might include up to 20 % (mol.) of α-palmito-β-palmitoleo-α'-stearin	28–29

The terms "olein" or "oleo"- indicate that linoleic as well as oleic radicals are concerned in these cases.

SUMMARY.

1. The chief component glycerides of the hen body fat studied are about 40 % (mol.) of monopalmitodi-"oleins", with nearly 30 % (mol.) each of mainly unsaturated tri-C_{18}-glycerides and of di-C_{16}-mono-C_{18}-glycerides. Fully-saturated glycerides (mainly tripalmitin) only form about 2 % of the fat. The proportion of stearic acid (*ca.* 7 %) combined in the fat may be present in the form of monostearo-glycerides in either of the three main groups; the combined amount of such monostearo-unsaturated glycerides will approximate to 20 % (mol.) of the fat. The individual distribution in the mixed glycerides of oleic and linoleic acids (present respectively to the extent of about 36 % and 21 % of the total com-

ponent acids) cannot be given, since the glyceride structure has been deduced mainly from study of the hen fat after partial or complete hydrogenation.

2. The 40 % of monopalmito-glycerides are almost certainly present in the form of β-palmitodi-"oleins" (or, to some extent, β-palmito-stearo-"oleins"). Consideration of the structure of the hydrogenated fats leads to the conclusions that the di-C_{16}-glycerides consist largely of α-palmito-derivatives, and that the 8 % of palmitoleic acid in hen fat is also probably mainly present in this group. The di-C_{16}-glycerides, on this basis, may be α-palmito-β-palmitoleo-α′-"olein", α-palmito-β-"oleo"-α′-palmitolein, α-palmito-β-palmitoleo-α′-stearin, or a mixture of these.

3. The hen body fats are differentiated from the corresponding fats of pigs, sheep, cows and some other animals by their unusually large proportions of tri-C_{18}-(unsaturated)-glycerides and, especially, of the mixed semi-unsaturated di-C_{16}-mono-C_{18}-glycerides. The result is a more heterogeneous mixture of mixed triglycerides than in fats of the lard type. This is reflected in the physical consistency of the fats. A pig fat of similar mean unsaturation and not very different palmitic, stearic, oleic and linoleic acid contents forms a thin, semi-solid, almost homogeneous paste at atmospheric temperature; but the hen body fats separate into two well-marked phases—a clear liquid fat with a lower layer of solid glycerides (so-called "stearin").

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REFERENCES.

Amberger and Bromig (1921). *Z. Unters. Nahr. Genussm.* **42**, 193.
Bömer (1922). *Z. Unters. Nahr. Genussm.* **43**, 101.
Hilditch, Jones and Rhead (1934). *Biochem. J.* **28**, 786.
—— and Stainsby (1935). *Biochem. J.* **29**, 90.

LXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLII. THE METABOLIC PRODUCTS OF *ASPERGILLUS TERREUS* THOM. A NEW MOULD METABOLIC PRODUCT—TERREIN.

BY HAROLD RAISTRICK AND GEORGE SMITH.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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THE name *Aspergillus terreus* is used by Thom and Church [1918; 1926] to cover a series of strains which have a number of well-marked morphological features in common, but which show a certain amount of variation in colour and type of growth. The conidial colour may range from pale cinnamon to fairly deep brown; the texture of the surface growth may vary from smooth velvety to definitely floccose, whilst the reverse of the colony and the agar may vary in colour from pale or bright yellow to fairly dark brown. Thom and Church state that *A. terreus* is common in soil and decaying vegetable matter throughout the United States, but has not been received by them from any European source. Smith [1928] found this organism as a latent infection of cotton yarns, occurring fairly often on American cottons and very frequently on all types of Egyptian cottons; also as a cause of serious damage to exported cloths, particularly dhooties.

The present communication is an account of experiments which have been carried out with five strains of *A. terreus*, obtained from different sources and covering approximately the range of variation shown by different strains in the series. When grown at 24° on the well-known Czapek-Dox solution with 5 % of glucose as the sole source of carbon, two of the strains, No. 45 and Ac 100, produce in the solution a new mould metabolic product which we have named *terrein* ($C_8H_{10}O_3$, M.P. 127°).[1] The yield of terrein appears to reach a maximum when the sugar in solution has been reduced to approximately 1 %. Succinic acid has also been identified as a metabolic product of No. 45, but is formed in small amounts only. From the metabolism solutions of the remaining three strains (No. 3, No. 37 and Ac 24) no terrein has been isolated but only small amounts of oxalic acid, succinic acid or both. The strain Ac 100 is of special interest since it produces, in addition to the new product terrein, appreciable quantities of citrinin, a substance hitherto believed to be formed only by *Penicillium citrinum* Thom [Hetherington and Raistrick, 1931]. The other four strains also give rise to fairly large amounts of substances which, like citrinin, are precipitated by acidification of the metabolism solutions. From none of these acid precipitates has any citrinin been obtained and so far no other substance has been isolated in a state of purity.

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[1] At a meeting of the Biochemical Society held at Oxford on May 5th, 1934, a paper was read in which this substance was described as "terreic acid" [Raistrick and Smith, 1934]. It has since been found that a highly purified sample has p_H 6·8 in 1 % aqueous solution, and work on the constitution of terrein, at present in progress, has shown that the substance does not contain a true acidic group. Hence the change of name.

Thom and Church [1918] state "With the accumulation of material however, we find ourselves with a series of related strains (of *A. terreus*) rather than a single organism....Whether some of them may ultimately be separated as varieties, upon physiological grounds, is not determined". We suggest that if as the result of future research the *A. terreus* series be subdivided into a number of named species, the production of terrein when grown on Czapek-Dox glucose medium at 24° be diagnostic for one of these named species.

EXPERIMENTAL.

The organisms.

Relevant details of the five strains of *A. terreus* used in this investigation are given in Table I.

Table I.

Catalogue no.	Source	Isolated by	Cultures on Czapek-Dox agar: Surface colour	Reverse colour	Texture
No. 3	Mildewed cloth	G. Smith 1926	Cinnamon	Deep yellow	Smooth velvety
No. 37	Egyptian cotton	G. Smith 1926	Cinnamon	Greenish yellow	Smooth velvety
No. 45	American cotton	G. Smith 1926	Brownish	Brown	Slightly floccose
Ac 21	British National Collection of Type Cultures No. 981	Thom and Church (Washington 144)	Brownish	Deep brown	Slightly floccose
Ac 100	F. T. Brooks, Cambridge	Hansford. Identified by Miss Church	Brown	Deep brown	Definitely floccose

Cultural conditions.

The culture medium used throughout was the well-known Czapek-Dox solution containing: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; distilled water, 1000 ml. Cultures were grown in conical flasks of 1 litre capacity, each containing 350 ml. of medium, and sown, after sterilisation, from cultures on Czapek-Dox agar slopes approximately 2 weeks old, one slope being used to sow 4 flasks. The temperature of incubation was 24°.

The course of metabolism.

Twenty flasks of medium were sown with each of the 5 strains, and single flasks were taken for analysis at intervals of 3 or 4 days. The experiment was continued for 35 days, by which time metabolism appeared to be complete in all cases. The examination included measurement of p_H colorimetrically, estimation of apparent residual glucose by polarimeter, estimation of bromine absorption

Table II.

R.S.P., % apparent residual sugar determined by polarimeter. B.A., bromine absorption in mg. Br per ml. Initial p_H, 4·1; initial glucose, 5·17 %; initial bromine absorption, 0·08 mg. Br per ml.

Days' incubation	No. 3			No. 37			No. 45			Ac 21			Ac 100		
	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.
4	5·3	4·72	0·10	6·4	4·82	0·16	6·5	4·68	0·14	5·2	4·57	0·51	6·4	1·74	0·51
7	4·3	3·37	0·43	6·8	3·26	0·35	6·6	4·29	0·64	4·8	3·96	1·74	6·7	4·05	0·83
10	4·8	2·56	0·30	6·9	2·26	0·53	6·4	3·13	1·23	4·5	2·91	1·98	6·8	3·48	1·73
13	4·6	1·32	0·46	6·5	2·15	0·58	6·5	1·23	1·18	4·5	2·28	2·14	5·4?	1·53	1·92
17	5·6	0·36	1·34	6·4	1·35	0·64	6·4	1·41	4·22	5·0	1·26	3·01	6·6?	1·58	2·86
21	5·3	0·44	0·93	6·2	0·64	0·98	7·0	0·77	3·84	5·1	0·81	3·32	6·3?	0·96	4·09
24	6·5	0·31	1·15	6·2	0·28	1·68	6·6	0·69	3·76	5·2	0·69	3·46	6·3?	0·56	3·34
28	7·5	0·05	1·86	6·1	0·22	1·78	7·4	0·32	4·90	—	0·33	4·19	6·5?	0·30	4·41
32	7·3	0·05	2·00	6·5	0·19	1·84	7·8	0·62	5·41	—	—	3·36	—	0·10	3·88
35	8·4	0·05	2·56	7·3	0·15	2·10	7·3	0·23	4·30	—	0·18	3·50	—	0·14	4·77

by Koppeschaar's method [1876] and observation of the effect of adding $FeCl_3$ solution. After 2 weeks' incubation the reactions of the metabolism solutions with various metallic salts were tested. The results of the analyses are given in Table II.

With the strains No. 45, Ac 24 and Ac 100 the metabolism solution gradually became coloured an intense reddish brown. Estimation of p_H was thereby rendered difficult and, in the later stages of incubation, with Ac 24 and Ac 100 was quite impossible. For estimation of residual glucose the solutions from No. 45 and Ac 100 could be cleared by addition of a few drops of HCl and filtration. With Ac 24, however, this treatment did not appreciably lighten the colour and the figures are therefore only approximate.

The figures show that with all the strains there was some irregularity in growth, so that single flasks, though selected to be as representative of the whole as possible, could not be regarded as averages of a whole batch. With No. 3, No. 37, No. 45 and Ac 100 the appearance of the cultures was reasonably uniform. Ac 24 however showed great irregularity. In some flasks the solution remained yellow for a long time and the mycelium was thick, gelatinous and non-sporing. In others the solution quickly became intense brown, with the mycelium thinner, brittle and sporing freely.

The metabolism solutions of No. 3 and No. 37 did not at any stage give an appreciable reaction with $FeCl_3$. With No. 45, Ac 24 and Ac 100, $FeCl_3$ intensified the brown colour of the solutions and, with Ac 100 after 17 days, gave a precipitate. After 13 days' incubation solutions from all the strains reduced $AgNO_3$ rapidly in the cold, gave slight brownish precipitates with $HgCl_2$, $CuSO_4$ and uranium acetate, and heavy precipitates with neutral or basic lead acetate. Acidification gave precipitates in all cases, greatest in amount with No. 45 and Ac 100, and increasing with continued incubation.

Large-scale experiments.

For isolation and identification of products of metabolism each strain was grown in batches of 100 flasks. When the glucose was reduced to the desired figure (about 1 %) as determined by testing single pilot flasks, the combined metabolism solution was poured off and filtered. The mycelium was scraped out of the flasks, well pressed and washed and the filtered squeezings and washings added to the main filtrate. The whole was made slightly acid to Congo red by cautious addition of strong HCl (about 100 ml.) and the precipitate allowed to settle. The clear supernatant liquid was decanted, the residual precipitate filtered, well washed and dried *in vacuo* (fraction I). The supernatant liquid and filtrate were combined and evaporated *in vacuo*, with a bath temperature not exceeding 50°, to approximately 750 ml. Usually a further quantity of flocculent precipitate separated during evaporation, also a certain amount of black tar (fraction II) which has not been further examined. The concentrate was centrifuged (filtration was impracticable) until almost clear and the precipitate added to fraction I. The aqueous solution was exhaustively extracted with equal volumes of ether, 20 to 30 extractions being usually necessary. On evaporation the ether extracts gave fraction III. Only small amounts of syrupy material were obtained by subsequent extraction with $CHCl_3$.

Strain No. 3. Fraction I was a brown powder, weighing 29·4 g. from which no crystalline material has yet been obtained. The ether extracts, fraction III, gave on evaporation small amounts of crystals (1·34 g.) which were identified as oxalic acid by M.P., mixed M.P. and the usual chemical tests. Further evaporation of the ether mother-liquor gave only small amounts of reddish gum.

Strain No. 37. Fraction I was a bright yellow powder weighing 30 g. Extraction with ether gave thick syrups from which no crystalline material has been obtained. Ether extracts, fraction III, gave traces of oxalic acid and appreciable quantities of succinic acid, identified by analysis, M.P. and mixed M.P., amounting in one experiment to 8·5 g.

Strain No. 45. Fraction I was a buff-coloured powder, the weight of which in six successive experiments is given in Table III. It was extracted in a Soxhlet apparatus with several successive lots of ether until nothing further was dissolved, this requiring about 24 hours in all. The extracts, which were dark brown in colour, deposited on standing hard crusts of microcrystalline material which were filtered off and well washed with ether. Evaporation of the mother-liquors gave further amounts of apparently identical material, and was repeated until nothing but a thick, almost black syrup remained. The solid isolated was yellowish buff in colour and had M.P. 193–196° (decomp.). No satisfactory method of purification and no reliable criteria of purity have yet been found.

The ether extracts (30 extractions with an equal volume of ether were carried out) containing fraction III, on evaporation to small volume deposited long needles of crude terrein (fraction III*a*). The first ten ether extracts were combined. They were dark-coloured but gave clean, pale-coloured crystals, whilst extracts 11–30, which were also combined, were much paler but gave brownish crystals mixed with small amounts of sticky syrup. The ether mother-liquors were further evaporated, those from extracts 11–30 giving further small amounts of crude terrein (fraction III*a*), whilst those from extracts 1–10 gave crystals which softened at 115°, became red at 145–150° and finally melted about 170° (fraction III*b*). The crude terrein constituting fraction III*a*, M.P. 124–126°, was recrystallised from ether, in which it is only sparingly soluble, until of constant M.P., 127°. The pure material forms fine colourless needles, which are very difficult to free from the last traces of colour. Fraction III*b* was obviously a mixture, and was shown to consist of terrein (which is readily detected in mixtures on account of its reddening at 145–150°) and succinic acid.

In order to prove the presence of the latter, the mixture was dissolved in a minimum of water and excess of neutral lead acetate added. The precipitate was filtered off, well washed, suspended in water and decomposed by passing H_2S. The PbS was filtered off and the clear aqueous solution evaporated to dryness. The crystalline residue had M.P. 184°, unchanged on mixing with authentic succinic acid. This method does not give a complete separation, although it does result in the isolation of pure succinic acid, since lead succinate is not quantitatively precipitated unless the solution is first neutralised, and neutralisation is undesirable because of the instability of terrein. Terrein, still somewhat impure, was recovered by extraction of the lead acetate mother-liquors with ether.

The yields of products from six batches of Strain No. 45, each of 100 flasks, are given in Table III.

Table III.

Batch no.	Days' incubation	R.S.P. %	Crude precipitate (g.) Fraction I	Pure terrein (g.)	Mixture of terrein and succinic acid (g.)
1	29	0·4	46	8·5	2·76
2	33	0·2	52	9·3	0·31
3	26	0·7	38	13·1	3·70
4	27	0·5	38	13·0	0·54
5	26	0·38	80·5	4·5	1·64
6	23	0·9	31	17·3	2·05

Strain Ac 24. The acid precipitate (fraction I) was a brown powder, weight 22·9 g. The only crystalline product so far obtained from the ether extracts (fraction III) was succinic acid, 2·8 g. from 100 flasks.

Strain Ac 100. The acid precipitate, fraction I, was a yellowish green powder, weight 57·5 g. Extraction with ether gave a dirty yellow, micro-crystalline powder which melted at 170°, and decomposed in a manner characteristic of citrinin. Recrystallised from absolute alcohol it was obtained as long golden yellow needles, M.P. 173° (decomp.), mixed M.P. with an authentic sample of citrinin from *P. citrinum* Thom, 172° (decomp.). In absolute alcohol solution ($c = 0{\cdot}92$), $[\alpha]^{23^\circ}_{5461} - 43{\cdot}1^\circ$. (Hetherington and Raistrick [1931] give for citrinin $[\alpha]_{5461} - 41{\cdot}7^\circ$, $-43{\cdot}9^\circ$.) Titration in aqueous suspension gave equiv. wt. 245, and in alcoholic solution 254. Estimation of molecular weight by depression of F.P. of dioxan gave 259. (Found (Schoeller): C, 62·36, 62·25; H, 5·59, 5·68 %. $C_{13}H_{14}O_5$ requires C, 62·37; H, 5·64 %. Mol. wt. 250.) A solution of the sodium salt gave on addition of $FeCl_3$ a heavy buff-coloured precipitate, soluble in excess of the reagent to give the clear deep iodine-brown solution characteristic of citrinin.

The statement by Hetherington and Raistrick [1931] that citrinin was apparently produced only by *Penicillium citrinum* Thom and was to be regarded as diagnostic for this organism, was based on the fact that no other of the hundreds of moulds tested at that time gave the typical reaction given by *P. citrinum* when $FeCl_3$ is added to the metabolism solution. Although the strain of *A. terreus* Ac 100 was actually one of the strains examined by Hetherington and Raistrick it is not surprising that citrinin was not detected by its colour reaction with $FeCl_3$, since this is almost entirely masked by the intense colour of the untreated metabolism solution and by simultaneous reactions between $FeCl_3$ and other metabolic products.

Because of the importance of this outstanding exception, the only one encountered in 13 years' work, it seemed desirable to repeat the experiment with *A. terreus* Ac 100 after rigorously re-checking the purity of our cultures. To this end the culture which was used as the starting point for the first 100-flask experiment with Ac 100 was plated out and showed no sign of contamination. Further, single spore cultures were made and these were used to sow the flasks in a second experiment. Citrinin was again obtained. There can thus be no doubt that citrinin is a metabolic product of at least one authentic strain of *A. terreus* Thom.

The yield of citrinin could not be estimated accurately since, as shown by Hetherington and Raistrick, it cannot be recovered quantitatively from very impure crude samples. In the first experiment 5·5 g. of pure material were obtained. In the second experiment, in which the crude acid precipitate was much darker in colour, only 3·5 g. were isolated.

Ether extracts of the metabolism solution, fraction III, gave crude terrein on evaporation. It was purified by recrystallisation from ether until practically colourless and of sharp and constant M.P. In the first experiment referred to above, final glucose 0·50 %, 4·8 g. of pure terrein were obtained. In the second experiment referred to above, final glucose 1 %, 6·1 g. of pure terrein were obtained. No succinic acid was isolated in either experiment.

General properties of terrein.

The substance crystallises from ether or acetone in colourless needles, M.P. 127°. The melt resets instantly on cooling and melts again at the same temperature. On heating to higher temperatures it begins to turn yellow at 145°, without any other visible change. The colour deepens as the temperature rises, becoming deep red at about 175°, and if held for some time at this temperature the melt does not solidify on cooling.

(Found (Schoeller): sample *ex* ether, C, 62·37, 62·33; H, 6·59, 6·53 %; OCH_3, nil; N, nil. Sample *ex* acetone, C, 62·33; H, 6·46 %. $C_8H_{10}O_3$ requires C, 62·31; H, 6·54 %.)

Mol. wt., by depression of F.P. of dioxan = 157·5. $C_8H_{10}O_3$ requires 154·1.

Optical rotation. In 1 % solution in water $[\alpha]_{5461}^{20^\circ} + 185^\circ$, $[\alpha]_{5790}^{20^\circ} + 168{\cdot}5^\circ$. In 2 % aqueous solution $[\alpha]_{5461}^{21^\circ} + 183{\cdot}4^\circ$, $[\alpha]_{5790}^{21^\circ} + 167{\cdot}2^\circ$.

Active hydrogen (Roth). In anisole at 28°, 1·60, 1·67 atoms; at 95°, 1·85, 1·84 atoms; in pyridine at 28°, 2·06 atoms of active hydrogen.

Solubilities at room temperature were estimated approximately by adding the desired solvent from a micro-burette to 0·1 g. of finely powdered substance and shaking vigorously until solution was just complete. Solubilities, as g. substance in 100 ml. of solvent are: water, 11·9; alcohol, 10·5; acetone, 5·75; chloroform, 0·75; ether, 0·32; benzene, 0·02; light petroleum, nil.

Reactions. A 1 % aqueous solution is neutral to litmus and has p_H 6·8. Addition of NaOH turns the solution first yellow, then brown and finally dark brown on standing. The neutral aqueous solution gives no reactions with salts of Ag, Ca, Ba, Hg″, Cu″, Fe‴, U, Ni or Pb. Fehling's solution is reduced instantly in the cold, bromine is rapidly absorbed without giving a precipitate, acid $KMnO_4$ is instantly decolorised, and Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl) produces a turbidity almost immediately and gives a bright red precipitate on standing overnight. An alkaline solution of terrein reduces $AgNO_3$ rapidly in the cold, reduces iodine instantly and immediately discharges the colour of alkaline phenolphthalein. The constitution of this interesting substance will be the subject of a future communication.

SUMMARY.

1. Five strains belonging to the group species *Aspergillus terreus* Thom have been grown at 24° on Czapek-Dox solution with glucose as sole source of carbon, and their metabolic products have been examined.
2. Two of the five strains give rise to a new mould metabolic product *terrein*, $C_8H_{10}O_3$, M.P. 127°, the isolation and properties of which are described.
3. One of these strains produces, in addition to terrein, citrinin, $C_{13}H_{14}O_5$, the crystalline yellow colouring matter which has previously been obtained only as a metabolic product of *Penicillium citrinum* Thom.
4. Of the three strains that do not produce terrein, one gives succinic acid, one oxalic acid, while the third strain gives a mixture of these two acids.

Our best thanks are due to Dr A. E. Oxford who kindly carried out for us determinations of the molecular weights of terrein and citrinin.

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REFERENCES.

Hetherington and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 153.

Koppeschaar (1876). *Z. anal. Chem.* **15**, 233.

Raistrick and Smith (1934). *Chem. Ind.* **53**, 451.

Smith (1928). *J. Text. Inst.* **19**, T 92.

Thom and Church (1918). *Amer. J. Bot.* **5**, 85.

—— —— (1926). The Aspergilli. (London: Baillière, Tindall and Cox.)

LXXII. POLYSACCHARIDES SYNTHESISED BY MICRO-ORGANISMS.

I. THE MOLECULAR STRUCTURE OF MANNOCAROLOSE PRODUCED FROM GLUCOSE BY *PENICILLIUM CHARLESII* G. SMITH.

BY WALTER NORMAN HAWORTH, HAROLD RAISTRICK AND MAURICE STACEY.[1]

From The Chemical Laboratories, University of Birmingham and the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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IT has recently been shown that, using glucose as the sole source of carbon, a number of hitherto undescribed metabolic products are formed when *Penicillium Charlesii* G. Smith is grown on Czapek-Dox solution of the sugar [Clutterbuck *et al.*, 1934]. Among the products are two new polysaccharides one of which consists only of mannose residues and the other of galactose residues. In addition, six hitherto unknown organic acids are found. The present communication describes the investigation of the molecular constitution of the polymannose to which is given the name *mannocarolose*. This is a white, amorphous, hygroscopic powder, $[\alpha]_{5780} + 66^\circ$, readily soluble in water and forming an acetate which is soluble only in organic solvents. Repeated fractional precipitation of the polysaccharide and removal of ash by electrodialysis yielded a product which was essentially homogeneous and on hydrolysis this gave a yield of 97 % of mannose, isolated as the phenylhydrazone. The molecular weight of the acetate was calculated by the use of the Staudinger factor from the viscosity measurements and corresponded to 9 mannose units in the molecule. This value is in agreement with that determined by the estimation of the mean iodine number. The same value is also found by a direct determination or gravimetric assay of the end group of the methylated polysaccharide. The latter yielded, on hydrolysis, 13·4 % of tetramethyl-methylmannoside which corresponds to a chain length of 8–9 mannose units. It is of considerable interest to record the general agreement of all three methods of determining the molecular size of the polymannose.

The precise form in which mannose is combined in the polysaccharide whether as pyranose or furanose, and also the particular mode of linking of adjacent units of the hexose were investigated in the following way. Methylated mannocarolose was completely hydrolysed, the hydrolysis products converted into the glycosides, and a quantitative analysis of the products revealed the presence of 75 % of a trimethyl-methylmannoside (I), 13·4 % of tetramethyl-methylmannoside, together with another fraction (10 %) which was identified as mainly a dimethyl-methylmannoside. On complete methylation and hydrolysis, the trimethyl- and dimethyl-mannosides yielded 2:3:4:6-tetramethylmannopyranose (III) which was characterised as its crystalline anilide.

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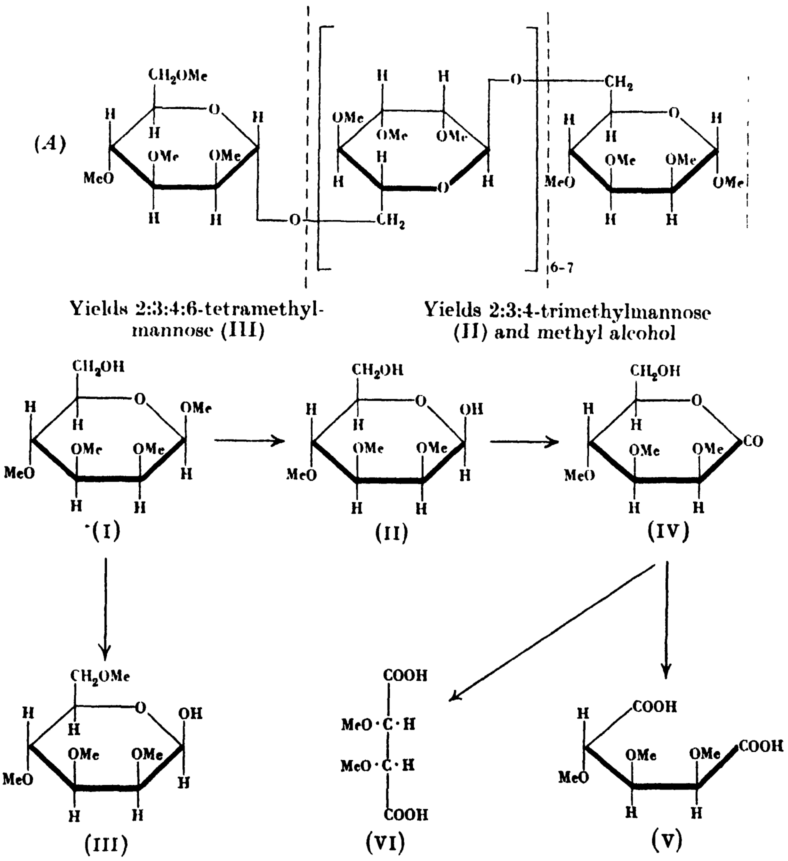
[1] Beit Memorial Research Fellow.

The trimethylmannose fraction was identified as a crystalline 2:3:4-trimethylmannopyranose (II), M.P. 102–103°, $[\alpha]_{5780}^{21^\circ}+7^\circ$ (equilibrium value) which is now described for the first time. The positions of the methyl groups were determined and the experimental evidence for the allocation of the 2:3:4-positions is here given. The trimethyl sugar gave, on oxidation, 2:3:4-trimethylmannopyranolactone (IV) which was distilled; its hydrolysis curve in aqueous solution and the rate of lactonisation of the corresponding acid were characteristic of a δ-lactone of the mannose series. It gave on further methylation a quantitative yield of tetramethylmannopyranolactone characterised as its crystalline phenylhydrazide. It was evident therefore that the hydroxyl group at position 4 in the trimethyl-sugar must have been methylated. This was shown conclusively by the degradative oxidation of the trimethylmannonolactone (IV) with nitric acid, which yielded *d*-arabotrimethoxyglutaric acid (V) and *i*-dimethoxysuccinic acid (VI) both of which were characterised as their crystalline methylamides [Goodyear and Haworth, 1927]. The isolation of the former of these products proves that the methylated positions in the trimethylmannose


(A)
CH₂OMe
MeO
CH₂
6-7
Yields 2:3:4:6-tetramethylmannose (III)
Yields 2:3:4-trimethylmannose (II) and methyl alcohol
CH₂OH
(I)
(II)
(IV)
CO
CH₂OMe
(III)
COOH
MeO·C·H
MeO·C·H
COOH
(VI)
COOH
(V)

must be at hydroxyls 2, 3, 4; that position 5 must be engaged in ring-formation; and that the terminal position 6 is to be represented as a free hydroxyl group liberated from the methylated polysaccharide by fission during hydrolysis. It is evident therefore that mannocarolose is constituted by the linking of mannopyranose units at positions 1 and 6 and that the form of mutual linking of successive units of mannose characterises the structure of the polysaccharide. This mode of union of hexose units differs fundamentally from that found in starch and cellulose in which the glucose residues are united at positions 1 and 4.

In the original hydrolysis of methylated mannocarolose it was noted that about 10 % consisted of a dimethyl-sugar which on glycoside formation was identified as 2:3-dimethyl-methylmannoside in the following way. It yielded on further methylation 2:3:4:6-tetramethyl-methylmannopyranoside identified by hydrolysis and isolation of the crystalline anilide. The 2:3-dimethylmannose gave on oxidation a γ-lactone which was further degraded by oxidation with nitric acid and yielded only crystalline *i*-dimethoxysuccinic acid, characterised also as its crystalline methylamide.

In the hydrolysis products of methylated mannocarolose this 2:3-dimethylmannose occurs to the extent of 10 % corresponding very nearly with the amount of the 2:3:4:6-tetramethylmannose which was isolated as the non-reducing terminal group. It should be noted that the polysaccharide reduces alkaline hypoiodite and gives an iodine number corresponding to the presence of one reducing group in the chain length of 9 mannose residues. The formulation can hardly be represented therefore by a continuous loop with a tetramethylmannose projection as a side-chain. The absence of reduction to Fehling's solution is in contrast to the hypoiodite reaction. The occurrence of one dimethyl residue of the 9 which otherwise have their quota of methyl groups in the methylated polysaccharide may be significant in other directions, but at the moment we incline to the view that inasmuch as it is not possible to ensure complete homogeneity of methylated mannocarolose by a procedure corresponding to that of crystallisation it seems inevitable that any not quite completely methylated portion may account for the occurrence of this quantity of dimethylmannose. The formulation (A) is subject to this modification, and although the stereochemical form is represented as that of α-mannose it should be clearly understood that this is not based on anything more definite at the moment than the comparatively high dextrorotation (+66°) of the polysaccharide.

EXPERIMENTAL.

The crude mixture of mannocarolose and galactocarolose (*i.e.* the polygalactose) was prepared by the method described by Clutterbuck *et al.* [1934]. The yields and properties of batches on Czapek-Dox medium and on Raulin-Thom medium were consistent with those previously described. Moreover by the method of fractionation it was shown that the crude polysaccharide mixture obtained from the Czapek-Dox medium contained mannocarolose and galactocarolose identical in properties with, but different in amount from, those obtained from Raulin-Thom medium as was previously suggested [Clutterbuck *et al.*, 1934, p. 103]. About equal amounts of mannocarolose and galactocarolose were isolated from the polysaccharide mixture from the Czapek-Dox medium, whereas with the Raulin-Thom medium the proportions were 30 % and 70 % respectively.

A sample of mannocarolose, prepared as previously described, was subjected to rigorous purification to attain homogeneity. The last portions (1 %) of ash were difficult to remove by alcohol precipitations, but all traces were removed by electrodialysis during 18 hours. The product had the following properties: it was a white hygroscopic powder, $[\alpha]^{21^\circ}_{5780} + 66^\circ$ (*c*, 3·3) in water. (Found: C, 44·8; H, 6·8 %. $(C_6H_{10}O_5)_x$ requires C, 44·4; H, 6·2 %.) Its solution was neutral to litmus and did not reduce Fehling's solution. After acid hydrolysis it gave mannosephenylhydrazone in 97 % yield and crystalline *d*-mannose in 84 % yield. The latter compound was crystallised from glacial acetic acid and the mother-liquors yielded mannosephenylhydrazone (12 %). Mean iodine number [Bergmann and Machemer, 1930], 14·3, corresponding to a molecular weight of 1400.

Mannocarolose acetate.

Mannocarolose (20 g.) was acetylated as previously described [Clutterbuck *et al.*, 1934]. The mannocarolose acetate (OAc, 46·1 %) was a fine white powder, easily soluble in chloroform and acetone, slightly soluble in ethyl alcohol and insoluble in methyl alcohol, ether and light petroleum. It was separated into four fractions by the graded addition of light petroleum to a 25 % chloroform solution. Each fraction showed identical properties. M.P. 150–160°; $[\alpha]^{20°}_{5780}+53°$ in acetone (*c*, 1·6), $[\alpha]^{20°}_{5780}+66°$ in chloroform (*c*, 0·84). Ash, 0·07 %. (Found: C, 50·1; H, 5·8; OAc, 46·0 %. $(C_{12}H_{16}O_8)_x$ requires C, 50·0; H, 5·6; OAc, 44·8 %.) The mean iodine number was 8·0, corresponding to a molecular weight of 2500.

Viscosity measurements were carried out on each fraction and the values obtained were identical. The molecular weight calculated by the use of the Staudinger factor from the viscosity measurements was 2584, corresponding to 9 mannose units in the molecule, a value which supports that calculated from the mean iodine number of the original polysaccharide and similar to that determined by the gravimetric assay of the end-group of the methylated polysaccharide.

Methylated mannocarolose.

(1) *By direct methylation of mannocarolose.* In a typical experiment mannocarolose (5 g.) was dissolved in water (20 ml.) and methylated in the usual manner by the gradual addition of sodium hydroxide and dimethyl sulphate during 5 hours. After 30 minutes acetone (100 ml.) was added. The temperature was maintained at 35–40° during the addition of the reagents, after which it was raised to 80–90° until all the acetone was removed (30 minutes).

Partly methylated mannocarolose was obtained as a light brown gum on the surface of the hot solution, and it redissolved on cooling. The temperature was raised and the methylated material was separated and dissolved in chloroform. The solution was dried over anhydrous magnesium sulphate and cleared by centrifuging. The chloroform was removed by distillation and the resulting thin syrup poured into light petroleum (B.P. 40–50°). The methylated product (5 g.) was precipitated as a fine cream-coloured powder. $[\alpha]^{20°}_{5780}+80°$ in chloroform (*c*, 1·0). (Found: OMe, 36·2 %.)

The product (5 g.) was re-methylated in acetone solution (50 ml.) at 40° with dimethyl sulphate and sodium hydroxide over a period of 2 hours. Before raising the temperature to 90° for the final heating the excess alkali was carefully neutralised and the solution diluted, since it was observed that the product was appreciably soluble in hot alkali. The methylated product separated as a brown gum and was fractionally precipitated as before from chloroform solution giving a light-brown powder (yield 5·4 g.). $[\alpha]^{20°}_{5780}+96°$ in chloroform (*c*, 1·2). (Found: OMe, 41·0 %.)

The product (5 g.) was now dissolved in methyl iodide (50 ml.) containing dry methyl alcohol (2 ml.), and re-methylated by heating gently for 4 hours with silver oxide (10 g.) which was added in 2 g. lots. The product was extracted with boiling chloroform, which was removed by distillation. On pouring the residual thin syrup into light petroleum (B.P. 40–50°) the methylated compound was precipitated as a light brown gum, prolonged trituration of which with renewed amounts of light petroleum (B.P. 40–50°) converted it into a cream-coloured powder. M.P. 95–100°. Yield 5·4 g. $[\alpha]^{21°}_{5780}+102°$ (*c*, 1·4) in chloroform. (Found: OMe, 44·4 %.) Further methylation by either method failed to alter the methoxyl content or the specific rotation.

(2) *By methylation of mannocarolose acetate.* Mannocarolose acetate (15 g.) was dissolved in acetone (200 ml.) and methylated at 45° by the simultaneous addition of dimethyl sulphate (140 ml.) and 30 % sodium hydroxide (290 ml.) in the usual manner. Acetone was added from time to time to retain the methylated product in solution. After the addition of the reagents (90 minutes) the temperature was raised to 80° for 30 minutes. The excess alkali was neutralised with dilute sulphuric acid and the cold solution poured slowly into ethyl alcohol (1·5 l.) and kept overnight. The separated sodium sulphate was filtered off and extracted with alcohol. The combined alcoholic solution and extracts were concentrated *in vacuo* to a viscid syrup which was dissolved in water (20 ml.) and re-methylated with dimethyl sulphate (100 ml.) and 30 % sodium hydroxide (210 ml.) in the presence of acetone at 35–40°. The product was isolated as previously described as a fine light brown powder. Yield, 10 g. $[\alpha]^{18°}_{5780}+80°$ (*c*, 2·0) in chloroform. (Found: OMe, 36·1 %.)

The above product was re-methylated twice with silver oxide and methyl iodide and the resulting almost completely methylated mannocarolose was obtained in the form of a cream-coloured powder. Yield, 10·5 g. M.P. 95–100°; $[\alpha]^{18°}_{5780}+102°$ in chloroform (*c*, 2·0). (Found: OMe, 44·2 %.) It was observed that the specific rotations of the partially methylated fractions gave a good indication of their methoxyl contents.

Fractionation of methylated mannocarolose.

The different preparations of methylated mannocarolose having $[\alpha]^{21°}_{5780}+102°$ in chloroform and methoxyl content, 44·4 %, were mixed (20 g.) and separated into three fractions as follows. The combined material (20 g.) was boiled for 20 minutes with ether (200 ml.), which was decanted, and then for a further 10 minutes with fresh ether (200 ml.). A gummy residue (A) remained undissolved. To the mixed ether solutions was added light petroleum (B.P. 40–50°), (400 ml.) with vigorous stirring. An oily precipitate (B) separated and the clear mother-liquor was decanted after standing for an hour. Evaporation of the solvent left a gummy residue (C). Each fraction was obtained as a cream-coloured powder by trituration with light petroleum (B.P. 40–50°) and had the following properties:

Fraction	Yield (g.)	% OMe	$[\alpha]^{20°}_{5780}$ in chloroform	Apparent mol. wt. by viscosity	Mol. wt. (Rast)
A	6·2	44·3	+102°	1960	2010
B	9·8	44·5	+102·5°	1960	1926
C	3·8	44·1	+101°	1960	2000

These results indicate that fully methylated mannocarolose prepared either by method 1 or 2 is fairly homogeneous. This compound was readily soluble in cold water, acetone, chloroform, methyl and ethyl alcohols, slightly soluble in ether and insoluble in hot water and light petroleum. (Found: C, 53·1; H, 7·8; OMe, 44·4 %. $(C_9H_{16}O_5)_x$ requires C, 52·9; H, 7·9; OMe, 45·6 %.)

Hydrolysis of methylated mannocarolose.

Methylated mannocarolose is comparatively stable towards acid. It was unaffected by 8 % methyl alcoholic hydrogen chloride even after boiling for several hours, but was completely hydrolysed by fuming hydrochloric acid at 15° in 4 days, the specific rotation $[\alpha]_{5780}^{15^\circ}$ changing from $+71^\circ$ to $+5{\cdot}2^\circ$ (c, 1·0). 15·3 g. were hydrolysed in fuming HCl (150 ml.) at 15° for 4 days. There was considerable darkening in colour of the solution towards the end of the reaction. The excess acid was removed by aeration and the residual solution completely neutralised by the addition of barium carbonate. The solution was filtered after the addition of a little charcoal and the filtrate and washings made up to 500 ml. This colourless solution was extracted 8 times with chloroform (250 ml. each time). The combined chloroform extracts were dried over anhydrous magnesium sulphate, filtered and the chloroform removed by distillation, giving 3·1 g. of a viscid pale yellow syrup which was strongly reducing to Fehling's solution. $[\alpha]_{5780}^{15}+28{\cdot}6^\circ$ in methyl alcohol (c, 3·1). This syrup was converted into the methylmannoside by boiling with 3 % methyl alcoholic HCl for 12 hours, the product being isolated in the usual manner. Yield, 3·1 g. of a mobile non-reducing syrup (fraction a), $n_D^{16\cdot5}$, 1·4597; OMe, 56·4 %.

The chloroform-extracted aqueous solution was concentrated *in vacuo* at 40° to 100 ml. and then poured into 90 % ethyl alcohol (1 l.) and filtered. The alcoholic filtrate was evaporated to dryness and the residue extracted eight times with chloroform. This chloroform solution was combined with a chloroform extract of the solid barium chloride which had been collected by the above filtration and yielded on evaporation 12 g. of a colourless viscid syrup, n_D^{17}, 1·4740; $[\alpha]_{5780}^{18^\circ}+30^\circ$ (in MeOH). This syrup was converted into the methylmannosides in the usual manner. There were thus obtained 12 g. of a non-reducing colourless mobile liquid (fraction b), $n_D^{16\cdot5^\circ}$, 1·4640. (Found: OMe, 51·91 %.)

Further prolonged extraction with chloroform of the alcohol and barium chloride residues gave fraction c (0·8 g.) as a very viscid syrup, $[\alpha]_{5780}^{20}+27^\circ$ in methyl alcohol (c, 1·5), which was also converted into the methylmannoside. Yield, 0·8 g., n_D^{16}, 1·4735. (Found: OMe, 42·5 %.)

Fractional distillation of the methylated mannosides (fractions a, b, *and* c.)

Fraction b (12·0 g.) which consisted mainly of trimethyl-methylmannoside was distilled *in vacuo* from a Claisen flask and gave the following sub-fractions:

Sub-fraction	Pressure (mm. Hg)	Bath temperature	Yield (g.)	n_D^{17}
b_1	0·12	120–125°	2·0	1·4595
b_2	0·12	125–135°	6·0	1·4620
b_3	0·12	135–160°	2·6	1·4650
b_4	0·12	160–170°	0·8	1·4735

Sub-fraction b_1 was now mixed with fraction a (total weight, 5·1 g.) and distilled from a Claisen flask with a Widmer flask as a receiver for the first fraction, and gave the following sub-fractions:

Sub-fraction	Pressure (mm. Hg)	Bath temperature	Yield (g.)	n_D^{17}
a_1	0·09	115–125°	4·0	1·4585
a_1	0·09	125–135°	0·5	1·4610
a_3 (Still residue)	0·09	—	0·5	1·4690

Sub-fraction a_1 (4 g.) was now re-distilled very slowly through the Widmer column and gave the following sub-fractions:

Sub-fraction	Pressure (mm. Hg)	Bath temperature	Yield (g.)	n_D
First drop	0·09	115–118°	—	1·4535
a_4	0·09	115–120°	1·1	1·4560
a_5	0·09	120–125°	0·68	1·4560
a_6	0·09	125–128°	0·41	1·4560
a_7	0·09	128–135°	0·20	1·4565
a_8	0·09	135–150°	1·30	1·4620
a_9 (Still residue)	—	—	0·30	1·4620

The methoxyl content of sub-fraction a_4 was 58·08 %, and sub-fractions a_4 to a_7 had distilled without effective separation of constituents. Accordingly a thin spiral fractionating column was now used, leaving a space of 2 cm. between the top of the column and the side arm. The outside of the column was surrounded by a thick layer of asbestos paper. Sub-fractions a_4 to a_7 were combined (2·3 g.) and distilled as follows:

Sub-fraction	Pressure (mm. Hg)	Bath temperature	Yield (g.)	$n_D^{21°}$	% OMe
A_1	0·06	118–120°	1·71	1·4512	60·7
A_2	0·06	120–125°	0·30	1·4560	58·1
A_3 (Residue)	—	—	0·30	1·4610	51·5

The amount of tetramethyl-methylmannoside in sub-fraction A_2 was 0·15 g. Thus the total yield of tetramethyl-methylmannoside from sub-fractions A_1 and A_2 was 1·86 g. To this amount must be added a predetermined 10 % correction for losses so that the total yield of tetramethyl-methylmannoside was 2·046 g. from 15·3 g. of methylated mannocarolose, corresponding to 13·4 % of tetramethyl-methylmannoside and to a chain length of 8–9 units.

Analysis and identification of the 2:3:4:6-tetramethyl-methylmannoside.

The tetramethyl-methylmannoside from sub-fraction A_1 showed $[\alpha]_{5780}^{20°}+71°$ in methyl alcohol (c, 0·84), $[\alpha]_{5780}^{20°}+46°$ in water (c. 1·0). (Found: C, 52·9; H, 8·4; OMe, 60·7 %. $C_{11}H_{22}O_6$ requires C, 52·8; H, 8·9; OMe, 62·0 %.)

On treatment with $2N$ H_2SO_4 at 100° the tetramethyl-methylmannoside (1 g.) hydrolysed completely in 4 hours, the equilibrium rotation being $[\alpha]_{5780}^{17°}+4°$. The H_2SO_4 in the hydrolysate was removed by the addition of barium carbonate, the solution filtered, concentrated *in vacuo* and the products of hydrolysis isolated by extraction with chloroform: 0·9 g. of a syrup was obtained which was strongly reducing to Fehling's solution and had $n_D^{15°}$, 1·4631. This was treated with aniline in boiling alcohol in the usual manner and gave 2:3:4:6-tetramethylmannopyranose anilide in 85 % yield. It crystallised in long needles from a mixture of alcohol and light petroleum and had M.P. 142° alone or in admixture with an authentic specimen.

Analysis and identification of 2:3:4-trimethyl-methylmannoside.

Sub-fractions b_2, a_2, a_8, a_9, A_3, having B.P. at bath temperatures of 125–150° at 0·06–0·12 mm. and n_D, 1·4610–1·4620, were combined and re-distilled from a Claisen flask. The product distilled completely at 130–135°/0·08 mm. as a colourless mobile syrup, $n_D^{17°}$, 1·4620; $[\alpha]_{5780}^{20°}+58°$ in water (c, 1·25). (Found: C, 50·9; H, 8·8; OMe, 51·02 %. Trimethyl-methylmannoside $C_6H_8O_2\,(OMe)_4$ requires C, 50·9; H, 8·5; OMe, 52·5 %.)

Further methylation of 2:3:4-trimethyl-methylmannoside.

1 g. of re-distilled trimethyl-methylmannoside was methylated twice with silver oxide and methyl iodide in the usual manner and the product (1 g.), distilled at 0·08 mm. at a bath temperature of 115–118°, showed $n_D^{21°}$, 1·4515, and $[\alpha]_{5780}^{20°}$ $+42·5°$ (*c*, 0·83). This syrup was hydrolysed at 100° with 2*N* H_2SO_4, the rotation changing from $+42·5°$ to $+3·3°$ in 3 hours. 2:3:4:6-Tetramethylmannopyranose was isolated from the hydrolysis solution in the usual manner and gave 2:3:4:6-tetramethylmannopyranose anilide in 95 % yield, M.P. 142°, and identical with an authentic specimen.

Hydrolysis of 2:3:4-trimethyl-methylmannoside.

4 g. of trimethyl-methylmannoside were hydrolysed with 2*N* H_2SO_4 at 100 giving an equilibrium value of $[\alpha]_{5780}^{21°}$ $+6°$ after 3 hours. The hydrolysis product, 2:3:4-trimethylmannose, was extracted in the usual manner giving 3·8 g. of a colourless syrup having $n_D^{17°}$, 1·4735, $[\alpha]_{5780}^{21°}$ in water $+9°$ (*c*, 1·25) and OMe, 40·2 %. (Theoretical for $C_6H_9O_3(OCH_3)_3$, 41·9 %.) This syrup failed to give a crystalline anilide and gave an oily product on treatment in the usual manner with phenylhydrazine at 70° for 3 hours. The syrup crystallised on keeping in a desiccator for 3 months and was recrystallised from ether-light petroleum (B.P. 40–50°) in the form of triangular prisms, M.P. 94–96°, $[\alpha]_{5780}$ $+7°$ (equilibrium value) in water (*c*, 2·0). The M.P. 102–103° was higher when the substance was freshly crystallised as is the case with tetramethylglucose. (Found: C, 48·4; H, 8·3; OMe, 40·8 %. $C_6H_9O_3(OCH_3)_3$ requires C, 48·6; H, 8·1; OMe, 41·9.) Mutarotation in water (*c*, 20·4) zero hours $[\alpha]_{5780}^{20°}$ $+18·2°$; 5 min. $+15·3°$; 8 min. $+13·2°$; 17 min. $+9·1°$; 65 min. $+8·1°$; equilibrium value $+7·0°$.

Oxidation of 2:3:4-trimethylmannose.

2 g. of 2:3:4-trimethylmannose were warmed at 40° with bromine in aqueous solution for 48 hours and the lactone so formed was isolated in the usual manner. It was non-reducing to Fehling's solution and distilled at bath temperature 138–140° at 0·04 mm. Yield, 1·85 g. n_D^{21}, 1·4750, OMe, 41·7 %. (Theoretical for $C_9H_{16}O_6$, 42·3 % OMe.) Its hydrolysis curve in aqueous solution at room temperature was followed polarimetrically and was typical for the δ-lactone of the mannose series. Zero hours $[\alpha]_{5780}$ 158°; 17 hours, 122°; 22 hours, 110°; 38 hours, 105°; 48 hours, 103° (constant value). This behaviour is similar to that of a trimethylmannopyranolactone.

Further methylation of 2:3:4-trimethylmannopyranolactone.

0·5 g. of 2:3:4-trimethylmannopyranolactone was methylated with silver oxide and methyl iodide. The product was isolated by extraction with chloroform and had $[\alpha]_{5780}^{20°}$ $+154°$, changing to $+77°$ in 24 hours. This lactone was shown conclusively to be 2:3:4:6-tetramethylmannonolactone by the formation from it in quantitative yield of the characteristic crystalline phenylhydrazide, M.P. 184–185°, alone or in admixture with an authentic specimen.

Rate of lactonisation of 2:3:4-trimethylmannonic acid at room temperature.

Initial $[\alpha]_{5780}$ $+22°$; 18 hours, $+61°$; 42 hours, $+77°$; 66 hours, $+93°$; 80 hours, $+98°$ (constant value). The amounts of lactone and acid at equilibrium were approximately 43 % and 57 % respectively.

Oxidation of 2:3:4-trimethylmannopyranolactone.

2:3:4-Trimethylmannopyranolactone (1 g.) was oxidised by heating with nitric acid (sp. gr. 1·42) for 8 hours at 90°. The excess nitric acid was removed by distillation *in vacuo* with water for 6 hours and the product esterified with methyl alcoholic HCl. The esters (0·55 g.) were distilled as follows:

Fraction	Bath temperature	Pressure (mm. Hg)	Yield (g.)	$n_D^{16°}$	$[\alpha]_{5780}^{21°}$ in methyl alcohol
I	120–125°	0·06	0·31	1·4400	−9°
II	125–135°	0·06	0·13	1·4440	−22°
III	135–150°	0·06	0·10	1·4500	—

On nucleation with methyl *i*-dimethoxysuccinate, fraction I crystallised almost completely. The crystals after being drained on a tile and recrystallised from ether-light petroleum (B.P. 40–50°) had M.P. 68° alone or mixed with an authentic specimen of inactive methyl dimethoxysuccinate. Yield 0·1 g.

The non-crystalline portions of fractions I and II were treated with methylamine in dry methyl alcohol to form the methylamides. After keeping overnight, crystals in the form of long needles (0·07 g.) separated and were recrystallised from a mixture of ethyl acetate and ether; M.P. 210° alone or in admixture with an authentic specimen of inactive dimethoxysuccinomethylamide. The mother-liquors from the preparation of the methylamide were concentrated and crystallised completely (0·15 g.), M.P. 160–165°. Fractional crystallisation from ethyl acetate-ether gave 0·07 g. pure crystals, M.P. 171°, $[\alpha]_{5780}+58°$ in water (*c*, 0·56). This compound was shown to be *d*-arabotrimethoxyglutaromethylamide. It was found that an effective and less tedious separation of inactive dimethoxysuccinomethylamide from *d*-arabotrimethoxyglutaromethylamide could be effected by fractional sublimation of a mixture of the two substances either at ordinary pressures or *in vacuo*, the latter substance subliming at a much lower temperature than the former.

Analysis and identification of 2:3-dimethyl-methylmannoside.

Fraction *c* and sub-fractions a_3, b_3 and b_4 were combined and redistilled at a pressure of 0·08 mm. The fraction distilling with a bath temperature of 155–160° and weighing 1·5 g. had $n_D^{17°}$, 1·4735; $[\alpha]_{5780}^{21°}+64°$ (*c*, 1·25 in water). (Found: C, 49·4; H, 8·5; OMe, 41·1 %. Dimethyl-methylmannoside $(C_6H_9O_3(OCH_3)_3$ requires C, 48·6; H, 8·1; OMe, 41·9 %.) It was found by the procedure given below that the fraction consists mainly of 2:3-dimethyl-methylmannoside. On further methylation with methyl iodide and silver oxide, 2:3:4:6-tetramethyl-methylmannopyranoside was obtained, B.P. 120°/0·09 mm., $n_D^{21°}$, 1·4515. It gave 2:3:4:6-tetramethylmannopyranose anilide, M.P. and mixed M.P. 142° in 95 % yield. The 2:3-dimethyl-methylmannopyranoside gave, after hydrolysis with $2N$ H_2SO_4 at 100° for 3 hours, 2:3-dimethylmannose, $[\alpha]_{5780}^{21°}+16°$ in water (*c*, 1·2). (Found: OMe, 29·4 %. Theoretical for $C_6H_{10}O_4(OCH_3)_2$, 29·8 %.) It gave an oily osazone on treatment with phenylhydrazine. On oxidation of 0·8 g. of the 2:3-dimethylmannose with bromine at 40° for 24 hours it gave mainly 2:3-dimethylmannofuranolactone (0·6 g.), $[\alpha]_{5780}^{18°}+106° \rightarrow +66°$ in 14 days. This lactone on further oxidation with nitric acid (sp. gr. 1·42) yielded only *i*-dimethoxysuccinic acid, M.P. 68° (0·15 g.) characterised by the formation of its methylamide, M.P. 210°.

SUMMARY.

The molecular structure of mannocarolose, a polysaccharide produced from glucose by *Penicillium Charlesii* G. Smith, has been investigated. On acid hydrolysis mannocarolose gives *d*-mannose as the sole hydrolytic product.

Mannocarolose gives a methyl derivative which, on subsequent hydrolysis followed by glycoside formation, gives a mixture of 13·4 % of 2:3:4:6-tetramethyl-methylmannopyranoside, 75 % 2:3:4-trimethyl-methylmannopyranoside and 10 % of 2:3-dimethyl-methylmannopyranoside. Molecular weight determinations on mannocarolose and on its acetyl and methyl derivatives indicate that the chain length of mannocarolose is represented by 8–9 units of *d*-mannopyranose linked through the 1:6 positions. This is a unique molecular structure for a polymannose. Mannan A occurring in vegetable ivory has on the other hand the same structural linking as cellulose where the hexose units are joined at positions 1:4 [Klages, 1934]. Few of the naturally occurring mannans have been investigated and it is of interest that one form is present in the polysaccharide of the tubercle bacillus [Laidlaw and Dudley, 1925].

REFERENCES.

Bergmann and Machemer (1930). *Ber. deutsch. chem. Ges.* **63**, 316.
Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.
Goodyear and Haworth (1927). *J. Chem. Soc.* 3136.
Klages (1934). *Liebig's Ann.* **509**, 159.
Laidlaw and Dudley (1925). *Brit. J. Exp. Path.* **6**, 179

LXXIII. HISTAMINASE.

IV. FURTHER STUDIES IN PURIFICATION.

V. SOURCE OF THE AMMONIA FORMED IN THE HISTAMINE-HISTAMINASE REACTION.

By EARL WILLARD McHENRY and GERTRUDE GAVIN.

From the School of Hygiene, University of Toronto.

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In a previous communication in which attempts to purify histaminase were discussed, the authors [1932] showed that when this enzyme causes the destruction of histamine, ammonia is liberated stoichiometrically. In this paper the results of further studies on methods of purification of histaminase and on the histamine-histaminase reaction are presented.

As in previous studies [Best and McHenry, 1930; McHenry and Gavin, 1932] kidney powder was used as a source of histaminase. The amount of enzymic activity was determined by measuring the amount of histamine remaining after incubation for 24 hours at 38° in a mixture which originally contained a known amount of histamine and enzyme preparation. All determinations of histamine have been made by the blood-pressure method in etherised cats [Best and McHenry, 1930].

IV. FURTHER STUDIES IN PURIFICATION.

As a basis for these investigations histaminase solutions were prepared by extracting 1 part of kidney powder with 25 parts of Sørensen phosphate buffer, p_H 7·2 for one hour at 38°. A single extraction yields 60 % of the enzyme contained in the powder, and the yield is increased to 80 % by a second extraction. Longer extraction periods (2–24 hours) gave no greater yields.

The authors [1932] reported that the presence of phosphate ions augmented the activity of histaminase. If this were due to the precipitation of traces of calcium, which have been shown by Best and McHenry to be inhibitory, one might expect that oxalate ions would have the same effect. Extraction of kidney powder with 1 % sodium oxalate was as satisfactory as extraction with phosphate solution. Varying the concentration of oxalate (0–0·53 g. per 100 ml.) during the incubation of histamine with histaminase gave the same type of curve as was found for the effect of the concentration of phosphate on the enzyme. It is likely that the similar effects shown by the oxalate and phosphate ions are due to the removal of calcium presumably contained in the crude histaminase.

The effects on histaminase of a number of substances known to activate or inhibit enzymes have been tested. Preservatives such as 0·3 % tricresol and 0·1 % sodium benzoate completely inhibited the enzyme. Methylene blue (0·2 %) gave 60 % inhibition at p_H 7·2 and the effect was not changed by the presence of 0·005 *M* potassium cyanide. Glutathione 0·1 %, cystine 0·01 %, cysteine 0·015 %, ergothioneine 0·005 % and phenolindophenol 0·008 % had no apparent effect on histaminase.

Several protein precipitants, in addition to those previously employed, have been used in an effort to fractionate histaminase solution. 40 % of the potency was recovered in the precipitate when just sufficient picric acid to cause a precipitate was added. Precipitation from a mixture containing 0·1 % flavianic acid destroyed all activity. Precipitation from a mixture containing 0·5 % tannic acid and extraction of the precipitate with cold aldehyde-free acetone to remove the tannic acid, gave complete recovery of the enzyme. This process increases the activity twenty-fold on a dry-weight basis.

Earlier work had shown that an ammonium sulphate precipitate of the enzyme could be dried to give a stable powder. As a basis for subsequent experiments in purification a large quantity of this precipitate was prepared as follows. 30 g. of kidney powder were extracted, first with 750 ml. and then with 600 ml. of phosphate solution. The combined filtrates were stirred for one-half hour with 4 g. carbex E (Pfanstiehl) per 100 ml. of solution and filtered. This removes about 10 % of the impurities and causes very little loss of potency. Ammonium sulphate to give 90 % saturation was added to the filtrate, the precipitate filtered off by suction and dried in a vacuum-oven at a temperature under 50°. A phosphate buffer solution of this dried precipitate was used in the following experiments. Ice-cold alcohol may be used to precipitate the enzyme. When the concentration of alcohol was varied between 30–90 % the amount of enzyme precipitated varied directly with the concentration of alcohol, a maximum recovery of 65 % being obtained with 90 % alcohol. On a basis of unit activity per g. this method gave little purification. From an ice-cold mixture of 10 parts enzyme solution, 10 parts alcohol, 6 parts ether, the precipitate contained 75 % of the original activity. Ice-cold aldehyde-free acetone also precipitated the enzyme. The percentage potency recovered increased in proportion to the acetone concentration with a maximum of 65 % activity with 90 % acetone. When the acetone, prior to admixture, was neutralised with sodium hydroxide, 95 % of the activity was recovered in the precipitate if the concentration of acetone was between 60–80 %. On a dry weight basis this procedure gave no apparent purification as phosphate was precipitated by concentrations of acetone above 50 %. By dissolving the ammonium sulphate powder in distilled water neutralised with sodium hydroxide and by treating this solution with acetone, a four-fold increase in activity on a dry-weight basis was obtained. A phosphate solution of this precipitate was stable for one month at refrigerator temperature.

Experiments were made on adsorption of the enzyme from a phosphate solution of the kidney powder. Histaminase was not adsorbed by Eastman's norite, darco, special carbon, Pfanstiehl's carbex E, or Lloyd's reagent. Kaolin in water, water and alcohol, phosphate buffer, phosphate buffer and alcohol showed no adsorption of the enzyme. The most useful method was the aluminium hydroxide procedure developed by Sherman *et al.* [1930], for pancreatic amylase. The gel was prepared as described by these authors. It was found advantageous to have both phosphate and alcohol present during the adsorption phase. The best proportions were found to be 6 parts gel, 5 parts alcohol, 4 parts phosphate buffer, 15 parts histaminase solution. This mixture was stirred for 15 minutes, centrifuged, and the supernatant fluid, which contained no potency, was discarded. Very little enzyme was eluted with 1 % ammonium hydroxide or with 1 % diammonium hydrogen phosphate. Elution with phosphate buffer p_H 7·2 and 8·8 gave 75 % recovery, while at p_H 5·9 there was a 50 % recovery. Increasing the concentration of phosphate three times or decreasing it to one-third normal gave lower yields. The most successful method of elution was to use a mixture of phosphate buffers p_H 6·0 to 7·2 and glycerol. Yields of 85–90 % of the original activity were obtained when the glycerol concentration was between 40–60 %. If a phosphate solution of an ammonium sulphate powder was used in the above procedure only a 50 % yield was obtained. A still lower yield was secured if a solution of the acetone precipitate of the ammonium sulphate powder was used. Attempts to precipitate the enzyme after elution by tannic acid were unsuccessful.

V. SOURCE OF THE AMMONIA FORMED IN THE HISTAMINE-HISTAMINASE REACTION.

We have previously found that during the histamine-histaminase reaction the iminazole nucleus of histamine is ruptured and also that at p_H 7·2 one mol. of ammonia per mol. of destroyed histamine is liberated. If this ammonia were formed from the side chain, a change in the amino-nitrogen content during the reaction would be expected. In preliminary experiments to ascertain the source of the ammonia it was found that, owing to the presence of interfering substances in the solution, colorimetric methods of determining amino-nitrogen could not be used. Measurements with the Van Slyke gas analysis apparatus were more successful. With this method it was essential first to remove the proteins from the solution to be tested. The routine procedure of precipitation with sodium tungstate carried down a portion of the histamine present. Other protein precipitants, *e.g.* molybdic acid, tungstomolybdic acid, tannic acid, alcohol, zinc sulphate, magnesium sulphate, lead acetate, precipitated all or a part of the histamine. With Schenk's reagent and with copper sulphate no histamine was precipitated but the resulting solutions were unsuitable for use in the Van Slyke apparatus. The method finally adopted was precipitating with 1 volume of 10 % trichloroacetic acid to 1 volume of kidney solution and washing the precipitate with 5 % trichloracetic acid solution. For each experiment 3 flasks were used: *A*, histamine + a phosphate solution of histaminase p_H 7·2 + toluene; *B* and *C*, a similar histaminase solution + toluene. These were incubated for 48 hours at 38° to destroy the histamine in flask *A*. After incubation histamine equivalent to that originally present in flask *A* was added to flask *C*. Flask *B* served as a control for the amino-nitrogen value of the enzyme solution. The proteins in each flask were then precipitated with trichloroacetic acid. Immediately before the gas analysis the trichloroacetic acid filtrates were neutralised and histamine

Changes in amino-nitrogen during enzymic destruction of histamine.

		Before incubation		After incubation		
		Mg. histamine initially present	Mg. amino-nitrogen theoretical	Mg. residual histamine (cat assay)	Mg. amino-nitrogen theoretical	Mg. amino-nitrogen as found
Histamine inactivated (flask A)	(*a*)	109	14	0	0	1·1
	(*b*)	109	14	1	0·1	0·8
	(*c*)	109	14	4	0·5	3·1
	(*d*)	87	12	11	1·4	2·0
	(*e*)	109	14	20	2·6	2·9
Control (flask C). *Note.* Histamine added after incubation	(*a*)	109	14	56	7·3	6·6
	(*b*)	109	14	98	12·8	12·4
	(*c*)	109	14	102	13·2	13·1
	(*d*)	87	12	71	9·2	8·1
	(*e*)	109	14	92	11·9	10·9

assays were made by the blood pressure method in etherised cats. It will be seen in the following tables that when large amounts of histamine were inactivated there frequently remained a small amount of amino-nitrogen in excess of that due to the kidney solution and any remaining histamine. Some inactivation of histamine took place in the control flasks probably owing to the high temperature in the laboratory during the experiments.

From these results it is evident that there is a very marked fall in amino-nitrogen value during the destruction of histamine. This would indicate that the ammonia formed during the reaction is derived from the amino-nitrogen of the side chain and that the rupture of the iminazole nucleus yields no amino-compounds.

SUMMARY.

A number of procedures for the concentration of histaminase have been described. During the histamine-histaminase reaction the amino-nitrogen value falls almost to zero indicating that the ammonia liberated is derived from the side chain of the histamine molecule.

Grateful appreciation is expressed to Miss Neta Thompson for valuable assistance and to Dr C. H. Best and Dr J. G. FitzGerald for their continued interest in the investigation.

REFERENCES.

Best and McHenry (1930). *J. Physiol.* **70**, 349.
McHenry and Gavin (1932). *Biochem. J.* **26**, 1365.
Sherman, Caldwell and Adams (1930). *J. Biol. Chem.* **88**, 295.

LXXIV. A NEW TYPE OF MICROBURETTE.

BY NORMAN GEORGE HEATLEY.

From the Biochemical Laboratory, Cambridge.

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DURING the last decade several microburettes have been described, notably those of Benedetti-Pichler [1926], Kirk [1933], Linderström-Lang and Holter [1931; 1933], Rehberg [1925], Schilow [1927], Schwartz [1933] and Widmark and Orskov [1928]. Till a year ago, none of them, with the possible exception of that of Pincussen [1927], was specially designed for serial titrations. Two different types have been described during the last year, which are specially designed for this purpose.

That of Conway [1934] is simple, easy and cheap to make, and accurate. Though entirely suitable for the purpose for which it was designed, it can only be used with one solution.

The tapless microburette of Links [1934] is more complicated but it can be used with different solutions; it is not difficult to clean and has most of the advantages of the screw-controlled type. It can be used with any solution as the latter does not come into contact with the mercury.

The burette described below was designed before the description of either Links's or Conway's apparatus came to the attention of the author and had been, in fact, partly constructed. It is not as accurate as some other types, but as it has been in use for several months with satisfactory results, and as it can be made from the material usually available in the laboratory, its description may be of some interest.

Some of its main advantages are as follows:

1. The readings are taken at a mercury-air surface.
2. The titrating reagent only comes into contact with pyrex glass, paraffin wax and paraffin oil, and once prepared is never exposed to the air.
3. A large number of titrations can be carried out without refilling.
4. The titrating reagent can be changed almost instantaneously.
5. No cleaning is necessary when changing reagents.
6. Calibration is simple and direct.
7. The burette can be used for small quantities.

PRINCIPLE.

The apparatus is similar in principle to the measuring device of Hahn [1929]. The various titrating fluids are contained in small bottles, each of which is fitted with two tubes, a delivery tube (with a titrating point) and a second tube which connects the bottle to the burette proper. An exactly measured volume of liquid paraffin is displaced from the burette into the bottle, from which in turn an equal volume of titrating fluid is delivered.

CONSTRUCTION.

1. *Stock bottles.* For stock bottles, 2 oz. "Kali" bottles are chosen and coated inside with paraffin wax. An ordinary cork is bored to carry a 5 mm. tube, *H* which ends flush with the bottom of the cork, and a delivery tube of pyrex

glass bent twice at right angles and passing to the bottom of the bottle. The cork is boiled in paraffin wax for a time, then the tubes are fitted, and the cork is forced as tightly as possible into the neck of the bottle while the wax is still hot. The solution with which the bottle is to be filled is sucked up the delivery tube (by applying suction to *H*), and when it is nearly full, pure paraffin oil is poured down the tube *H* in such a way as to exclude all air.

2. *Burette proper.* The general scheme of the apparatus will be seen from Fig. 1. *B* is a length of graduated thermometer tubing, calibrated by weighing a thread of mercury of known length. It is connected at one end with the pressure-regulating mechanism *C*, and at the other, through the bulb *D*, with an ordinary three-way stopcock *E*. The second tube of the stopcock expands into the reservoir *F*, and the third is connected with a shallow U-shaped piece of capillary tubing *M*; to this is attached the tube *H* of the stock bottle. The reservoir *F*, tube *M*, tap *E*, and the upper half of the bulb *D* all contain paraffin oil. The lower half of *D* and the burette stem *B* contain mercury, as does the pressure-regulating mechanism *C*. When *C* is lowered, and *F* is connected to *D*, the mercury column in *B* will flow towards the zero mark; when *C* is raised, and the tap is turned through 180 degrees, the mercury will travel along the scale in the opposite direction and expel an equivalent volume of titrating fluid from the titrating point.

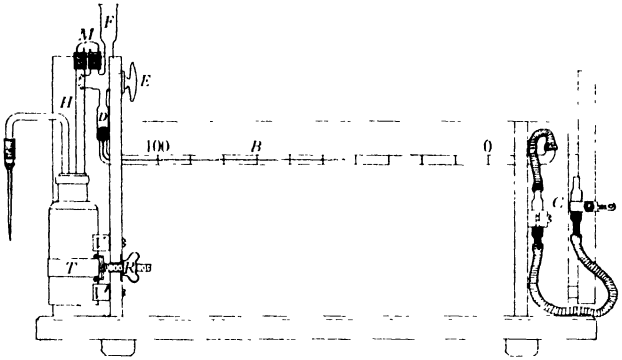


Fig. 1.

3. *Stand.* Absolute rigidity is essential for the proper functioning of the burette. This is obtained by clamping the stock bottle against the two V-shaped wooden chocks *V* and *V'*, by means of the flexible copper strip *T*, which is tightened by the butterfly nut *R*. (The blocks are allowed a certain amount of horizontal play to allow proper alignment of *H* with *M*, before tightening *R*.) The slide track, *etc.*, for the pressure-regulating mechanism was constructed from curtain rail fittings obtained from a sixpenny store.

4. *Titrating point.* This can be drawn from the delivery tube itself, though such an arrangement is extremely inconvenient if the tip gets damaged. A separate tip can, of course, be fitted with a properly ground joint, but after a certain amount of experiment, the joint described below has been found to be entirely satisfactory. The delivery tube of the stock bottle is first drawn out so that it will fit snugly into some pyrex tubing (Fig. 2, *a*). This latter tubing is then drawn out to a capillary from 4 to 6 inches long and is cut close above the drawn out part (Fig. 2, *b*); *a* is then moistened with water and dipped into carborundum powder, and the tip is roughly ground on. (It is not necessary to do more grinding than will prevent the sharp edges from splintering.) What grinding there is should be at the tip of *a* and not at the shoulder. Both parts are then thoroughly

cleaned and dried, and the titrating tip is drawn down to the requisite fineness. A blob of vaseline is placed on *a*, and *b* is gently warmed, so that on fitting it on to *a*, the vaseline will flow into the annular space between the two tubes. Finally a piece of rubber tubing which has been moistened inside with paraffin is pushed over the joint; it is easy to arrange that the rubber is in a state of tension, thus holding the tip firmly in position (Fig. 2, c).

The tip must be protected when not in use, and the titrating fluid must be prevented from syphoning over. This can be done in the following way. A piece of thin rubber tubing is placed over the shoulder of the titrating point so as to act as a cushion. The mouth of the pyrex tube (Fig. 2, *d*) is held against this, so as to make an airtight joint, by an elastic band which passes over the horizontal part of the delivery tube (this and the copper stirrup by which it is attached to the pyrex tube are not shown on the diagram). The protecting tube contains a small amount of titrating fluid covered by a layer of paraffin oil.

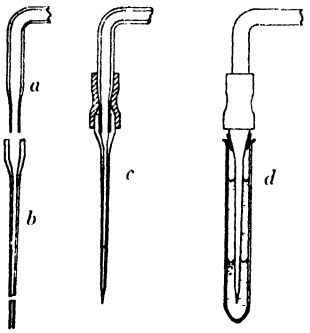


Fig. 2.

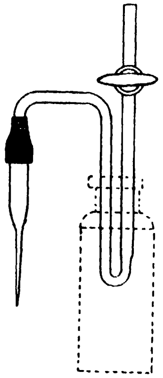


Fig. 3.

MANIPULATION.

The stock bottle is loosely clamped in position by the strip *T*, and the tube *H* and its rubber connection are filled with paraffin. (A small wash-bottle containing paraffin is convenient for this purpose.) Whilst the tube *M* is being pushed home, the tap *E* is momentarily opened. The butterfly nut is then tightened, and the protecting tube is removed from the titrating point. A small amount of the reagent is run out of the titrating point, and the mercury in *B* is brought back towards the zero mark by lowering *C* and connecting *D* to *F*.

The titration is carried out by raising *C* and turning the tap through 180 degrees. As there are no drainage errors, it does not matter how fast the first part of the titration is carried out; towards the end-point, however, *C* is lowered so that there is only a slight positive pressure, sufficient to expel the fluid from the titrating point only when the latter is immersed in the liquid being titrated. The fluid is prevented by surface tension from escaping when the tip is withdrawn. This principle has been used by Schilow, Schwartz, Linderström-Lang and others, and by its means the end-point can be very carefully controlled.

Use of burette for small samples.

Fig. 3 shows an arrangement which is used for solutions of which only a small quantity is available. In this case the bottle merely acts as a stand, the tube *H* being continuous with the delivery tube. It is used as follows: a short piece of pyrex tubing wide enough to fit over the delivery tube is drawn out to a titrating point. It is then filled with paraffin and attached to the delivery tube as shown by a rubber sleeve. The tube *H* is attached to the burette as usual, and the tap *P* is opened. The required fluid is then sucked up the titrating point by lowering *C*, the tap *E* being in the appropriate position. The titration is then carried out in the usual way and the titrating tip is discarded when finished with.

This attachment has been found useful when dealing with enzyme solutions *etc.*, for not only can the whole of the solution be used, but the exact volume of it is known before measuring it out. The ability to use all the available solution is not often an advantage, but as far as the author knows, such a property has not been claimed for any other microburette, though a number could easily be adapted for this purpose.

ACCURACY.

The burette in use has a scale approximately 25 cm. long and a capacity of 0·1 ml. It is divided into a hundred parts, each division representing 1·0 μl. To test the accuracy of the apparatus, twelve samples of approximately decinormal sulphuric acid were accurately measured out and titrated against decinormal NaOH from the burette, using methyl red as indicator. The following results were obtained:

Tube no.	Volume of *N* NaOH required (μl.)	Deviation from mean	% error
1	51·6	0·2	0·4
2	51·7	0·1	0·2
3	51·8	0	0
4	51·9	0·1	0·2
5	52·1	0·3	0·6
6	51·7	0·1	0·2
7	52·1	0·3	0·6
8	51·8	0	0
9	51·8	0	0
10	52·2	0·4	0·8
11	51·6	0·2	0·4
12	51·4	0·4	0·8

These results leave much to be desired, but it must be remembered that the scale is comparatively short, and in the above titrations, which were selected at random, only half of it was being used.

At first the tap *E* was lubricated with a paraffin-rubber composition, but it was found that this soon became softened and dissolved by contact with the liquid paraffin, and a certain amount of creep was liable to occur. This difficulty was got over by using an aqueous starch-glycerol lubricant, prepared as follows: 0·5 g. of Kahlbaum soluble starch is stirred up with 0·2 ml. of 5 % mercuric chloride solution and 0·7 ml. of water and heated in a boiling water-bath for 10 minutes; 0·5 ml. of glycerol is then added and stirred in whilst the mixture is still hot. It thickens considerably on standing and should not be used till 3 or 4 hours after preparation.

The rubber joints at each end of the tube *M* slowly swell and absorb paraffin. The actual rate of absorption has no disturbing effect on the titration, but the tubing, which of course is thick-walled, must be renewed when it becomes too loose fitting.

Note. A second burette has been constructed which is similar in all respects to the above, except that two calibrated tubes are incorporated instead of one. They are of 0·5 and 0·05 ml. capacity respectively and are connected to bulb *D* through a two-way tap, so that either can be used.

REFERENCES.

Benedetti-Pichler (1926). *Z. anal. Chem.* **73**, 200.
Conway (1934). *Biochem. J.* **28**, 283.
Hahn (1929). *Mikrochemie, Pregl-Festschrift*, p. 133.
Kirk (1933). *Mikrochemie*, **14**, 1.
Linderström-Lang and Holter (1931). *Compt. Rend. Trav. Lab. Carlsberg*, **19**, No. 4.
—— —— (1933). *Compt. Rend. Trav. Lab. Carlsberg*, **19**, No. 14.
Links (1934). *Mikrochemie*, **15**, 87.
Pincussen (1927). *Biochem. Z.* **186**, 33.
Rehberg (1925). *Biochem. J.* **19**, 270.
Schilow (1927). *Z. anal Chem.* **70**, 23.
Schwartz (1933). *Mikrochemie*, **13**, 1.
Widmark and Orskov (1928). *Biochem. Z.* **201**, 15.

LXXV. UNSATURATED ACIDS OF NATURAL OILS.

I. HIGHLY UNSATURATED ACIDS FROM OITICICA OIL (*LICANIA RIGIDA*).

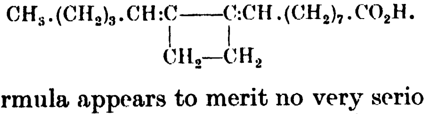
By WILLIAM BERNARD BROWN
AND ERNEST HAROLD FARMER.

From the Organic Chemistry Department, Imperial College of Science and Technology, South Kensington, London.

(*Received January 30th, 1935.*)

Oiticica oil comes into the market from Brazil in two forms, (1) as a fat of melting-point about 20° and (2) as a permanent oil. The botanical source of the oil has been for some years in doubt, being variously ascribed to the fruits of *Couepia grandiflora* (or *grandifolia*), *Licania rigida*, *Moquilea tomentosa*, *Pleragina umbrosissima*, *Arruda camara* and several other trees. The most recent opinion, however, ascribes the oil to the kernels of *Licania rigida*.

The authors' experiments were begun with the intention of investigating the constitution of the most highly unsaturated acid present (as a glyceride) in the oil of *Couepia grandiflora*, which according to the work of Van Loon and Steger [1931] is a conjugated triene acid, $CH_3.(CH_2)_3.(CH{:}CH)_3.(CH_2)_7.CO_2H$, isomeric with elaeostearic acid. If the constitution thus assigned were correct the new acid would add a third conjugated isomeride (supposedly of *cis-trans* type) to the two well-known forms (α- and β-) of elaeostearic acid.[1] The new acid, a substance very prone to atmospheric oxidation, has been designated by Van Loon and Steger couepic acid, and its constitution depends on its reputed conversion into stearic acid by catalytic hydrogenation (three double bonds) and its oxidation by ozone to yield valeric acid and azelaic acid. The physical characteristics of the acid are represented as being considerably different from those of α-elaeostearic acid: the optical exaltation, in particular, is low (2·3) in comparison with that of α-elaeostearic acid (8), a circumstance on which Rossmann [1932] subsequently bases a criticism of the triene constitution assigned by Van Loon and Steger, suggesting in place of the latter the non-conjugated semi-cyclic constitution



$$CH_3.(CH_2)_3.CH{:}\underset{CH_2-\!\!-\!\!-CH_2}{C-\!\!-\!\!-C}{:}CH.(CH_2)_7.CO_2H.$$

This semi-cyclic formula appears to merit no very serious consideration, but the multiplication of the isomerides of the economically important α-elaeostearic acid as reported by Van Loon and Steger—especially in view of certain recent experimental observations of Rossmann [1933] to the effect that elaeostearic acid passes on heating to 220° in an atmosphere of carbon dioxide into both a semi-cyclic isomeride and a dimeride—is of very great interest.

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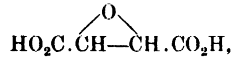
[1] Van Loon and Steger [1931] recognise five elaeostearic acids of which three (α-, β- and γ-) are stated to occur in chinawood oil, one (δ-) is obtained synthetically, and the fifth (ε-) is couepic acid.

The authors' attempts to obtain the authenticated fruits or oil of *Couepia grandiflora* proved unsuccessful and attention was turned to oiticica oil which appeared probably, although not certainly, to be the material actually employed by Van Loon and Steger. Two varieties of commercial oiticica oil and an adequate supply of oiticica kernels were obtained, the latter being definitely identified as kernels of *Licania rigida*. One of the commercial samples of oil was a semi-solid fat at room temperature, and this resembled the fat extracted by light petroleum from the disintegrated kernels (a faintly yellow oil until cooled); the other sample was permanently an oil and doubtless represented a "polymerised" oiticica oil which is obtained by heating the solvent-extracted or hot-pressed oil at 225° for about an hour. The permanently liquid oil, the heat-modified nature of which was not at first suspected by the authors, yielded on saponification a mixture of unsaturated and saturated acids from which the highly unsaturated component could readily be separated by crystallisation from petroleum. This acid, amounting to about 2 % of the total mixed acids (much of the original highly unsaturated acid having apparently undergone conversion into a petroleum-insoluble material), melted at 96·5° and rapidly absorbed oxygen from the atmosphere: it corresponded in all described characteristics with an acid, M.P. 94°, reported by Wilborn and Löwa [1929] to be isolable from *Couepia grandiflora*, but was apparently different from Van Loon and Steger's couepic acid (highest recorded M.P. 74–75°) in that it yielded on oxidation with permanganate not valeric acid and azelaic acid as principal products, as should a geometrical isomeride of elaeostearic acid, and as was indeed reported by Van Loon and Steger for their acid, but valeric acid and γ-ketoazelaic acid, $HO_2C.(CH_2)_4.CO.(CH_2)_2.CO_2H$. There was formed, however, in addition to the above-mentioned major products, a very small proportion of a dicarboxylic acid of M.P. 205°, doubtless fumarylglycidic acid


$$HO_2C.\overset{\diagup O \diagdown}{CH{-}CH}.CO_2H,$$

apparently derived by the dehydration of racemic acid.

Saponification of the two semi-solid fats yielded in each case a mixture of acids from which the highly unsaturated component could readily be isolated by crystallisation from petroleum. This substance was in each case the same, *viz.* an acid of M.P. 74–75°, which, although similar in general characteristics to the one from permanently liquid oiticica oil, showed a marked depression of melting-point on mixing therewith; furthermore in each case the acid was obtained in not less than 25 % (maximum isolated 33 %) yield, and in each case was unaccompanied by any considerable quantity of petroleum-insoluble acid. There was thus every indication that the semi-solid commercial oiticica oil was in reality licania oil in a state not seriously changed by the processes involved in its preparation. The subsequent observations are mainly concerned with the structure of what must now be regarded as the characteristic highly unsaturated acid of *Licania rigida*, and this acid is henceforth referred to as *licanic acid*. It is to be noted that the saturated and the less highly unsaturated acids of licania or oiticica oil have not been examined.

Licanic acid yielded on oxidation with permanganate valeric acid and a ketoazelaic acid identical with that obtained from the highly unsaturated acid of "polymerised" oiticica oil. These were the major products, but when oxidation was pushed well beyond the stage at which rapid decoloration of permanganate at 0° ceased, there were also obtained some oxalic acid (about $\frac{1}{3}$ mol.) and a quantity of syrupy and gummy acidic material which was susceptible of further slow oxidation with permanganate at room temperature. It is to be observed, however, that none of the dicarboxylic acid (M.P. 205°) referred to above was formed.

The ketoazelaic acid, which readily yielded a semicarbazone (M.P. 197°) and passed smoothly into azelaic acid on reduction by the method of Clemmensen, must necessarily be either the α- or γ-keto-derivative of the normal-chained dibasic acid, since it neither gave a colour with ferric chloride, as would a β-keto-acid, nor resembled the already-known δ-ketoazelaic acid (M.P. 108–109°; semicarbazone, M.P. 176–177°) synthesised by von Pechmann and Sidgwick [1904]. Its formulation as the γ-acid was confirmed by its degradation in the expected manner with Beckmann's chromic acid mixture; with this reagent it yielded as principal products adipic and succinic acids:

$$HO_2C.(CH_2)_4.\,\vdots\,CO\,\vdots\,.(CH_2)_2.CO_2H \rightarrow HO_2C.(CH_2)_4.CO_2H + HO_2C.(CH_2)_2.CO_2H.$$

In the presence of platinum licanic acid absorbed three molecular proportions of hydrogen yielding thereby hexahydrolicanic acid—an acid characterised by great stability towards permanganate but capable of forming a semicarbazone and possessing the empirical composition of a ketostearic acid. Thus, since both the two principal oxidation fragments are normal-chained acids, only two formulae offer themselves for the representation of licanic acid, *viz.* (I) and (II), which embody the two ways in which the ketoazelaic acid skeleton can be attached to the valeric acid skeleton whilst retaining triene unsaturation:

$$\text{(I)}\quad CH_3.(CH_2)_3.(CH{:}CH)_3.(CH_2)_4.CO.(CH_2)_2.CO_2H;$$
$$\text{(II)}\quad CH_3.(CH_2)_3.(CH{:}CH)_3.(CH_2)_2.CO.(CH_2)_4.CO_2H.$$

The correctness of the normal chain here depicted follows from the smooth conversion of the hexahydrolicanic acid into stearic acid by the method of Clemmensen:

$$\left.\begin{array}{ll}\text{(III)} & CH_3.(CH_2)_{13}.CO.(CH_2)_2.CO_2H \\ & \qquad\qquad\text{or} \\ \text{(IV)} & CH_3.(CH_2)_{11}.CO.(CH_2)_4.CO_2H\end{array}\right\} \rightarrow CH_3.(CH_2)_{16}.CO_2H.$$

Choice between the two formulae (III) and (IV) for hexahydrolicanic acid was made by oxidising the latter acid with chromic acid in acetic acid. The hexahydro-acid possessed a much higher degree of resistance towards chromic acid than did γ-ketoazelaic acid, but it broke down slowly on boiling with the oxidising mixture into a low-melting mixture of fatty acids and succinic acid; no adipic acid or glutaric acid was formed. The fatty acid product resembled myristic acid, but obviously was not homogeneous: doubtless it consisted of a mixture of myristic acid, $CH_3.(CH_2)_{12}.CO_2H$, and pentadecanoic acid, $CH_3.(CH_2)_{13}.CO_2H$, but, owing to the great difficulty of separating these acids, the isolation of the pure fatty acid components was not attempted with the amount of material at disposal. The dibasic acid complementary to pentadecanoic acid, *viz.* malonic acid would not survive under the conditions of oxidation. Of the two formulae (III) and (IV), III must therefore represent hexahydrolicanic acid; that is to say, hexahydrolicanic acid, M.P. 96·5°, is γ-ketostearic acid. Confirmation of this conclusion is supplied by the experimental establishment of identity between hexahydrolicanic acid and synthetic γ-ketostearic acid, M.P. 97°, the latter first obtained by Clutterbuck and Raper [1925] as an oxidation product of stearic acid and now obtained by repeating their preparation.

Licanic acid is accordingly γ-keto-$\Delta^{\theta,\kappa,\mu}$-octadecatrienoic acid (4-keto-$\Delta^{9:11:13}$-octadecatrienoic acid), represented by formula (I), and it is, so far as the authors are aware, the first ketonic polyene-acid outside the group of carotenoids to be described.

A point of interest arises in connection with the nature of the difference between the two triene acids described above. Both licanic acid and the acid from permanently liquid oiticica oil (*iso*licanic acid) are forms of γ-keto-$\Delta^{\theta\kappa\mu}$-octadecatrienoic acid, and the difference between the two would appear to be one of geometrical configuration about one or more of the double bonds. It is to be noted that only about half the quantity of acid taken readily undergoes degradation to valeric acid and γ-ketoazelaic acid, the remainder doubtless persisting as hexahydroxy-derivatives, which by reason of superior resistance to oxidative attack survive where their stereoisomerides undergo fission. The nature of these and of similar resistant polyhydroxy-acids obtained by oxidation of highly unsaturated acids is at present being investigated, but it is perhaps significant to find that although neither licanic nor *iso*licanic acid yields any appreciable quantity of racemic or mesotartaric acid, *iso*licanic acid gives a very small yield of fumarylglycidic acid (see p. 632).

Although there is nothing to show whether the oxide ring is formed during the oxidation process or subsequently by the dehydration of racemic acid, the appearance of the fumarylglycidic acid suggests that the middle double bond of *iso*licanic acid possesses a *trans*-configuration, in which respect it probably differs from licanic acid, which on further investigation may be found to yield the less-readily isolable maleylglycidic acid corresponding to mesotartaric acid.

It would seem likely that licanic acid is identical with Van Loon and Steger's couepic acid and that the latter authors' constitution for this acid is erroneous. There remains a possibility, however, that the botanical source of their *Couepia grandiflora* oil is a tree distinct from *Licania rigida*.

EXPERIMENTAL.

The triene acid from commercial oiticica oil (*liquid*). The liquid variety of oiticica oil as imported from Brazil was a dark golden yellow viscous liquid which from its permanent liquidity, comparatively high specific gravity (0·9898) and low iodine value (108·2) doubtless represented the so-called "polymerised" variety of oiticica oil, obtained by heating commercially expressed (or solvent-extracted) oil at 225°. The "polymerised" nature of the oil was not, however, known to the authors at the beginning of the work. The oil was saponified in the following way:

100 g. of oil were boiled under reflux for 5 hours with a solution made by dissolving 50 g. of caustic potash in the minimum quantity of water and diluting the product with 200 ml. of rectified spirit. The alcoholic solution of the potassium salts was made up to 500 ml. with cold water and then cooled in ice, cautiously acidified with 100 ml. of 45 % sulphuric acid (by weight) and finally shaken with 150 ml. of ether. The ethereal solution of fatty acids thus obtained was washed with water, dried with anhydrous sodium sulphate and gently warmed to remove the bulk of solvent; the free fatty acids were obtained by removing the residual solvent at reduced pressure without further heating.

The saponification product was immediately mixed with petroleum (B.P. 40–60°): at first a homogeneous solution was obtained, but on further addition of the solvent two layers separated, the lower layer consisting apparently of the partially oxidised or "polymerised" components of the commercial oil. This portion was twice extracted with petroleum but not further utilised. The combined upper layer and petroleum extracts were concentrated to a bulk of

300–400 ml. and allowed to cool to room temperature: a highly unsaturated acid crystallised out which was only sparingly soluble in petroleum. This acid was collected and twice extracted with 150 ml. of boiling petroleum (B.P. 40–60°), the extracts being rapidly filtered, united and allowed to cool. The crystals which then separated were collected, washed with petroleum and stored under petroleum to prevent atmospheric oxidation, which rendered them somewhat sticky in the course of several hours and ultimately converted them into a liquid material. One further recrystallisation was always carried out before the acid was used: in this way colourless flaky crystals of M.P. 96·5° were obtained. The maximum yield of acid was 1·3 g. from 100 g. of oil. Owing to the rather rapid absorption of oxygen the acid was troublesome to analyse but gave figures showing it to be an octadecatrienoic acid (C, 73·8, 73·4; H, 9·6, 9·65 %. $C_{18}H_{28}O_3$ requires C, 73·7; H, 9·65 %). On exhaustive hydrogenation at room temperature and atmospheric pressure, in an alcoholic medium and using a platinum catalyst, the acid took up three molecules of hydrogen and yielded a saturated acid which did not decolorise permanganate even on boiling. This saturated acid which crystallised from rectified spirit in colourless flakes of M.P. 96° (C, 72·95; H, 11·5 %. $C_{18}H_{34}O_3$ requires C, 72·75; H, 11·8 %) proved to be identical with the hexahydrolicanic acid described below; moreover the semicarbazone of the acid (M.P. 126°) and its methyl ester (M.P. 49·5°) proved on direct comparison to be identical with the corresponding derivatives of hexahydrolicanic acid described below.

On oxidation with approximately 3 % permanganate at 0° the unsaturated acid took up readily 7·3 atoms of oxygen. At this stage of oxidation the principal products were a volatile fatty acid recognised as valeric acid (B.P. 180–185°; *p*-bromophenacyl ester, M.P. 64°, mixed M.P. 64°) and a solid acid which after several recrystallisations from chloroform melted at 108–109°; there were also obtained some oxalic acid and a quantity of partly oxidised material. The acid of M.P. 108–109° had the composition and basicity of a ketoazelaic acid, subsequently identified as *γ-ketoazelaic acid* (C, 53·35; H, 6·9 %; mol. wt. (dibasic), 204. $C_9H_{14}O_5$ requires C, 53·4; H, 6·9 %; mol. wt., 202), and yielded a *semicarbazone* of M.P. 197° (C, 46·0; H, 6·6 %. $C_{10}H_{17}O_5N_3$ requires C, 46·3; H, 6·6 %). All the above-named oxidation products were also obtained from commercial oiticica fat and from the fat from *Licania rigida* kernels and are more fully described below; another oxidation product, however, was isolated from the crude ketoazelaic acid in very small yield, and this has been obtained from neither of the other varieties of oil studied. This substance, which differed greatly from ketoazelaic acid in respect of its sparing solubility in ether, its high M.P. (205° with decomp.), and high oxygen content, appears from its composition, basicity and general properties to be the fumarylglycidic acid described by Lossen [1906] (C, 36·75, 36·65; H, 3·25, 3·2 %; equiv., 65·9. $C_4H_4O_5$ requires C, 36·35; H, 3·05 %; mol. wt., 132). The further study of the liquid form of oiticica oil has been postponed.

The triene acid from commercial oiticica fat. The commercial fat was a cream-coloured solid consisting of a mixture of glycerides. Like the liquid variety of oil it was readily saponifiable by boiling for 5 hours with strong alcoholic potash, the resulting potassium soap yielding on cautious acidification with dilute sulphuric acid and extraction as described above a partly solidifiable mixture of fatty acids. From this mixture, which set on cooling to a pale yellow waxy solid, the highly unsaturated component was isolated by dissolving the former in boiling petroleum (B.P. 60–80°) and filtering off the crystals which separated on cooling. These crystals melted at once at 73–74°, the highest M.P. attainable even after numerous recrystallisations from petroleum being 74–75°. The crystals absorbed oxygen on exposure to the air yielding a sticky liquid; consequently it was convenient to preserve the once-crystallised acid under petroleum and crystallise it still once more immediately before use. The yield of this acid, which is henceforth designated *licanic acid*, amounted to 33 %.

On analysis licanic acid gave figures indicating that it possessed the empirical composition $C_{18}H_{28}O_3$ (C, 72·85; H, 9·65 %; mol. wt., ebullioscopic, in alcohol, 277; equiv., 291·5. $C_{18}H_{28}O_3$ requires C, 72·9; H, 9·65 %; mol. wt., 292) and on hydrogenation at room temperature and atmospheric pressure (platinum catalyst) in an alcoholic medium took up 3 mols. (experimental figure, 3·05 mols.) of hydrogen to yield a saturated acid of M.P. 96°. This *hexahydrolicanic acid* crystallised in colourless flakes from alcohol, the purest specimens obtainable melting at 96·5°; it possessed ketonic properties, yielding a well-defined *semicarbazone* which crystallised from aqueous alcohol in colourless flattened needles, M.P. 126° (C, 64·1; H, 10·25 %. $C_{19}H_{37}O_3N_3$ requires C, 64·15; H, 10·5 %); moreover its analytical figures pointed to the empirical formula $C_{18}H_{34}O_3$ (C, 72·70; H, 11·55 %; equiv., 295. $C_{18}H_{34}O_3$ requires C, 72·75; H, 11·8 %; mol. wt., 298). On esterification with methyl alcoholic hydrogen chloride, the hexahydro-acid yielded the corresponding *methyl ester* which crystallised from alcohol in colourless plates, M.P. 49–50° (C, 73·1; H, 11·6 %. $C_{19}H_{36}O_3$ requires C, 73·1; H, 11·6 %).

Thus licanic acid is a keto-octadecatrienoic acid, and direct comparison of this acid with the isomeric acid of M.P. 96·5° obtained from the permanently liquid variety of oiticica oil showed the two substances to be quite different: on the other hand the hexahydro-acids derived from the two triene acids proved to be identical in M.P. and in the characteristics of their derivatives; moreover there was no depression of M.P. on mixing the two hexahydro-acids, their semicarbazones or their esters, and both acids yielded identical products on oxidation with chromic acid (see below).

Reduction of hexahydrolicanic acid by Clemmensen's method. 0·7 g. of the hexahydro-acid, dissolved in 15 ml. of concentrated hydrochloric acid, was boiled for 4 hours with 6 g. of zinc amalgam, during which operation hydrogen chloride was passed into the boiling solution. The product, on precipitation with water and filtration, yielded a mixture of two acids, the components of which were readily separable by fractional crystallisation from petroleum (B.P. 60–80°) into unchanged hexahydrolicanic acid (M.P. 96°; mixed M.P. 96°) and stearic acid; the latter was identified by comparison with an authentic specimen (M.P. 69·5–70°; mixed M.P. 69·5–70°) and by conversion into its methyl ester (M.P. 36°; mixed M.P. 38°). Thus hexahydrolicanic acid is a ketostearic acid.

Oxidation of licanic acid. 19·5 g. of the acid dissolved in a solution of 10 g. of sodium carbonate in 100 ml. of water were oxidised at 0° with 1320 ml. of 4·85 % permanganate (9·1 atoms of oxygen). The manganese mud was filtered off and extracted three times with boiling water, the filtrate and aqueous extracts so obtained being united, concentrated to a bulk of 200 ml. and then acidified with slight excess of concentrated hydrochloric acid. The acidic product was extracted four times with 125 ml. portions of ether and the aqueous liquor first freed from ether and then from oxalic acid by precipitation with calcium chloride in presence of free ammonia; finally the ammoniacal filtrate was evaporated to dryness, the residue taken up in a little water and the filtered solution cautiously acidified and re-evaporated to dryness. From the final residue 3·2 g. of syrupy and viscous acidic material were extracted by ether and acetone used successively; a portion of this material esterified with methyl alcoholic hydrogen chloride yielded an oil which decomposed on distillation. On removal of the solvent the ethereal extract yielded a partly crystalline residue: the liquid portion of this yielded 4·6 g. of pure valeric acid of B.P. 184° (86–88°/15 mm.), the identity of which was confirmed by the preparation of the corresponding *p*-bromophenacyl ester of M.P. 64° (mixed M.P. with an authentic specimen, 64°); the rather sticky solid residue yielded on crystallisation from chloroform 6·1 g. of the γ-ketoazelaic acid (M.P. 108–109°; semicarbazone, M.P. 197°) previously obtained by the oxidation of permanently liquid oiticica oil and 2·9 g. of a syrupy acidic residue. It was remarkable that the crude calcium oxalate obtained from the oxidation product (about 3 g.) contained no considerable quantity of the salts of mesotartaric or racemic acid, although the restriction of the quantity of permanganate to that actually employed might have been expected to lead to their formation.

A similar oxidation carried out with 37·5 g. of licanic acid yielded 12·6 g. of ketoazelaic acid, 5·9 g. of valeric acid, 3·6 g. of calcium oxalate and 4 g. of brown syrupy acid; a portion of the last on re-oxidation with permanganate yielded a further small quantity of ketoazelaic acid.

Constitution of ketoazelaic acid. 1 g. of ketoazelaic acid was boiled for 4 hours with 10 g. of amalgamated zinc and 15 ml. of concentrated hydrochloric acid through which hydrogen chloride was bubbled continuously. On extraction with ether the cooled product yielded an acid which on crystallisation from water formed colourless prisms, M.P. 105–106°. This acid was different from the original acid, depressing the melting-point thereof and yielding no semicarbazone: it was identified as azelaic acid (mixed M.P. 105–106°).

3·5 g. of ketoazelaic acid were boiled gently with 45 ml. of Beckmann's chromic acid mixture until the latter was completely reduced. On exhaustive extraction of the cooled product with ether 4·2 g. of dry acids were obtained. Preliminary examination of this mixture by fractional crystallisation showed it to contain succinic acid, probably adipic acid and a substance melting below 100°. The mixture was therefore dissolved in water, rendered alkaline with ammonia and the boiling solution treated with calcium chloride; from the calcium succinate precipitated 0·2 g. of pure succinic acid, M.P. 185° (mixed M.P., 185°), was obtained. The dry mixed acids (3·2 g.) recovered from the ammoniacal liquor consisted mainly of adipic acid and yielded on crystallisation from ether-chloroform slightly impure adipic acid which on one further recrystallisation from the same medium attained the M.P. of 148° and gave no depression of M.P. with an authentic specimen of adipic acid. The small acidic residue (M.P. 95–100°) recovered from the solvent yielded no glutaric anhydride on treatment with acetic anhydride and appeared to be somewhat impure ketoazelaic acid.

From the above evidence it follows that the ketoazelaic acid obtained from licanic acid is the γ-keto-compound, a conclusion which is in agreement (*a*) with the observation that this ketoazelaic acid gave no colour with ferric chloride in aqueous or alcoholic solution, and (*b*) with the fact that this acid differed in melting-point and in the melting-point of its semicarbazone from the δ-ketoazelaic acid described by von Pechmann and Sidgwick [1904].

Oxidation of hexahydrolicanic acid. 2·8 g. of the hexahydro-acid were dissolved in 25 ml. of glacial acetic acid and thereto 5·12 g. of chromic oxide (twice the calculated quantity) were added. The mixture was boiled for 3 hours on a sand-bath and then cooled. After diluting somewhat and filtering off unchanged acid the filtrate was saturated with sulphur dioxide, boiled to expel excess of this reagent, diluted and treated with excess of ammonia. The chromium hydroxide precipitated on boiling the alkaline liquor was filtered off and the filtrate evaporated to dryness. The residue was acidified with slight excess of concentrated hydrochloric acid and the product extracted several times with ether; the aqueous liquor was evaporated to dryness and the residue extracted first with ether and then with acetone. From the first ethereal extract, after evaporation of the solvent and the acetic acid contained therein, there was obtained a semi-solid mass, from which light petroleum removed a low-melting solid acid, leaving a small quantity of unchanged hexahydrolicanic acid; the low-melting acid (M.P. 25–30°), doubtless largely myristic acid contaminated with some proportion of pentadecanoic acid, was not further examined owing to the improbability of its complete purification being effected with the amount of material at disposal. The second ethereal extract and the acetone extract both yielded nearly pure succinic acid, melting at 185° on recrystallisation (mixed M.P. 185°). Since no trace of adipic acid was isolated the ketone group of hexahydrolicanic acid must be in the γ-position, the hexahydro-acid being accordingly γ-ketostearic acid.

The triene acid from the oil of Licania rigida *kernels.* The disintegrated kernels on extraction with petroleum (B.P. 60–80°) in a Soxhlet apparatus gave a 26·3 % yield of a clear golden yellow oil; when extracted with ether a 35·8 % yield of a rather turbid pale yellow oil was obtained. Both products when cooled in ice set to a faintly yellow-coloured fat which retained its solidity at room temperature. On saponification of the oil with alcoholic potash licanic acid, M.P. 74–75°, was obtained in a yield rather less than that from commercial oiticica fat. The identity of this acid with the acid from the commercial oil was fully confirmed by its behaviour on oxidation and hydrogenation and also by the identity of the corresponding derivatives of the two acids.

Preparation of hexahydrolicanic acid from stearic acid. By repetition of the oxidative procedure described by Clutterbuck and Raper [1925] γ-ketostearic acid was obtained in the form of colourless flakes, M.P. 96°; this acid yielded a semicarbazone of M.P. 126°. The acid and its semicarbazone were found by direct comparison to be identical respectively with the hexahydro-acid derived from each of the above-mentioned specimens of oiticica oil and its semicarbazone.

SUMMARY.

Two specimens of commercial oiticica oil have been examined in respect of the most highly unsaturated acid contained therein. Oiticica fat yields by saponification a solid triene acid (M.P. 74–75°), the constitution of which has been shown by degradative and synthetic methods to be γ-keto-$\Delta^{\theta\kappa\mu}$-octadecatrienoic acid, $CH_3.(CH_2)_3.(CH:CH)_3.(CH_2)_4.CO.(CH_2)_2.CO_2H$. This acid is found to be identical with that obtainable by saponification of the freshly extracted oil from the kernels of *Licania rigida*. Thus the source of oiticica oil, which has long been in doubt, is almost certainly *Licania rigida*, and the name licanic acid is now applied to the above-mentioned acid.

From the second or permanently liquid variety of oiticica oil another form of γ-keto-$\Delta^{\theta\kappa\mu}$-octadecatrienoic acid (M.P. 96·5°) has been obtained in small yield. This appears to differ from its isomeride in geometrical configuration only and is designated *iso*licanic acid. Since the permanently liquid oil appears to have been heat-altered during its commercial preparation, the *iso*-acid is probably all that remains of the original licanic acid, the remainder having been oxidised, cyclised or polymerised.

Licanic acid appears to be the first naturally-occurring ketonic unsaturated

acid to be described. It is in all probability the same compound as the "couepic acid" of van Loon and Steger.

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REFERENCES.

Clutterbuck and Raper (1925). *Biochem. J.* **19**, 385.
Lossen (1906). *Liebig's Ann.* **348**, 261.
von Pechmann and Sidgwick (1904). *Ber. deutsch. chem. Ges.* **37**, 3817.
Rossmann (1932). *Rec. Trav. Chim. Pays-Bas*, **51**, 248.
—— (1933). *Fettchem. Umschau*, **40**, 96, 117.
Van Loon and Steger (1931). *Rec. Trav. Chim. Pays-Bas*, **50**, 936.
Wilborn and Löwa (1929). *Farben-Ztg.* **35**, 388.

LXXVI. INDOLE FORMATION BY *BACTERIUM COLI.*

I. THE BREAKDOWN OF TRYPTOPHAN BY WASHED SUSPENSIONS OF *BACTERIUM COLI.*

By DONALD DEVEREUX WOODS.

From the Biochemical Laboratory, Cambridge.

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Kuhne [1875] recognised indole as one of the products of the bacterial decomposition of proteins. The discovery of tryptophan by Hopkins and Cole [1901] was followed by the proof by these authors that tryptophan was the parent substance of bacterially formed indole [Hopkins and Cole, 1903]. They succeeded in isolating indole and indoleacetic acid from the products of the aerobic growth of bacteria (including *Bact. coli*) on a medium consisting of pure tryptophan and gelatin. Under anaerobic conditions they were able to isolate indolepropionic acid.

Hertzfeldt and Klinger [1915] studied, in roughly quantitative manner, the relationship between the disappearance of tryptophan and the production of indole with both indole-positive and indole-negative organisms. They used peptone medium and also a medium of pure tryptophan and salts, in which tryptophan was the sole source of nitrogen. In the latter case it is noteworthy that a form of bacterial suspension was used. Raistrick and Clarke [1921] investigated the bacterial decomposition of both the side chain and ring of tryptophan, but *Bact. coli* was not used. Fellers and Clough [1925] made a careful survey of the tests available for the detection of indole and skatole in bacterial cultures and applied them to a large number of bacterial species, but peptone and similar complex media were employed.

Bact. coli was chosen for the following work since it is a strongly indole-positive organism. An additional advantage is that *Bact. coli* does not decompose indole [Hertzfeldt and Klinger, 1915].

Experimental.

Most of the previous work on indole production has been done by means of growth experiments; it was decided here to employ washed bacterial suspensions for the following reasons. (*a*) The reaction occurs much more rapidly—about 36 hours instead of 3–4 days. (*b*) Pure tryptophan may be used as substrate and the reaction carried out in phosphate buffer, thus avoiding the complications incident to the use of bacterial media, such as the production of volatile phenolic substances. (*c*) Possible use of the tryptophan for bacterial growth is avoided. (*d*) Indole is the only volatile product.

In order to determine whether the indole produced could be recovered and estimated quantitatively from the bacterial suspension the following trial experiment was carried out. Distillation *in vacuo* of the cultures was tried in place of the steam-distillation, direct distillation or extraction with organic solvents previously used in this class of work. 2 ml. of stock *Bact. coli* suspension were added to 10 ml. of indole solution (containing 0·01–0·1 mg. indole per ml.). 10 ml. of this mixture were adjusted to p_H 9·2 [Zoller, 1920], using phenolphthalein as internal indicator, and washed into a small Claisen flask with 10 ml. water. This was distilled *in vacuo* almost to dryness, the tip of the condenser dipping below 10 ml. of water in an ice-cooled receiver. Two further amounts of 10 ml. of water were run into the flask and treated similarly. The temperature of the bath was not allowed to exceed 40°. The distillate and washings were made up to 100 ml. and were kept at 0° till estimated.

The distillation method of indole recovery has been criticised [Berthelot, 1914] on the grounds that some indole derivatives, *e.g.* β-indolecarboxylic acid decompose to indole on boiling. A number of indole derivatives (β-indolecarboxylic acid, β-indoleacetic acid, β-indolepropionic acid, β-indolepyruvic acid, β-indoleacrylic acid) have been submitted to distillation *in vacuo*, but no indole was detected, the lower temperature of distillation preventing any possible decomposition.

From a number of colorimetric methods available for the quantitative estimation of indole, that of Herter and Foster [1905], modified by Bergeim [1917] was chosen after trial. It has the advantage that the coloured compound formed is stable, and that the reaction is specific, no colour being given with skatole. Ammonia gives a green colour, but blank experiments in which culture distillates were shaken with synthetic permutit before estimation proved that sufficient ammonia to interfere was never present.

50 ml. of the distillate and 50 ml. of standard indole solution were run into separating funnels and to each were added 0·5 ml. of freshly prepared 1 % sodium β-naphthoquinonemonosulphonate and 1 ml. of 10 % potassium hydroxide. After standing for 15 minutes the blue solution was extracted successively with 5 ml. and 3 ml. of chloroform. The orange-red chloroform solution was run into 10 ml. volumetric flasks and made up to volume. The unknown and standard solutions were compared in a colorimeter with micro-plungers and cups (1 ml.). With concentrations of indole above 5 ml. of stock standard in 100 ml. the solutions were diluted with their own volume of water before estimation.

Stock standard. About 10 mg. of indole were weighed accurately, dissolved in a minimum volume of ethyl alcohol and diluted to 100 ml. with water. The solution remained constant in strength for 4–5 days if kept at 0°. Standard solutions of required strength were prepared from this stock standard.

Table I gives the percentage recovery of added indole from *Bact. coli* suspensions using the above method.

Table I.

Indole added (mg.)	Indole recovered (mg.)	% recovered	Indole added (mg.)	Indole recovered (mg.)	% recovered
0·927	0·901	97·2	0·515	0·500	97·1
0·927	0·888	95·8	0·515	0·492	95·6
0·927	0·895	96·5	0·206	0·199	96·4
0·721	0·684	94·9	0·206	0·197	95·7
0·721	0·695	96·4	0·103	0·100	97·3
0·515	0·499	96·9	0·103	0·097	94·5
				Mean	96·2

Preparation of bacterial suspensions. Bact. coli was grown on Roux bottles containing tryptic caseinogen broth agar. After 18 hours' incubation the growth of six bottles was washed off in the usual way with sterile precautions, washed and centrifuged twice with sterile Ringer's solution and finally suspended in 60 ml. of Ringer's solution and stored at 0°. The dry weight of 1 ml. suspension was 0·024 ± 0·003 g.

Tryptophan. The tryptophan used in this work was prepared from caseinogen according to Hopkins and Cole [1901], as modified by Dakin [1918]. It gave the following analytical figure:

Nitrogen % (micro-Dumas) 13·6 % Theoretical 13·7 %.

Indole formation from tryptophan.

In the following experiments small quantities of tryptophan were used since:

(*a*) the reaction is slow and if larger amounts are used a much longer time is needed for the completion of the reaction;

(*b*) it has been reported [Kilborn *et al.*, 1928] that 0·1 % of indole is toxic to *Bact. coli*. The concentration of indole formed in the experiments to be described did not exceed 0·01 %.

In view of the differing products obtained by Hopkins and Cole from the action of *Bact. coli* on tryptophan according to the degree of aeration, it was desirable to maintain good aerobic conditions. Air was bubbled through the suspensions at a steady rate. For each experiment the following quantities were used: 100 ml. tryptophan solution in phosphate buffer p_H 7·2 containing 20 mg. of tryptophan; 20 ml. stock *Bact. coli* suspension. The tryptophan solution was sterilised by filtration through a Seitz filter, and the apparatus was also sterilised. The solutions were run in with sterile precautions. The air current was turned on and the apparatus incubated at 37°. 10 ml. samples were removed aseptically at intervals and the indole formed was estimated by the method described. The p_H was checked with each sample and was found to remain constant.

In Fig. 1, curves *a*, *b* and *c* show some typical results obtained. Curve *d* shows the negligible autolytic production of indole when *Bact. coli* suspensions are incubated with water instead of tryptophan solution. Between 85 and 90 % of the theoretical yield of indole was obtained, though the time taken to reach this maximum varied between 16 and 36 hours. The yield of indole began to fall after reaching the maximum owing to volatilisation and the continuous aeration. 100 ml. of a solution of indole (of strength chemically equivalent to the

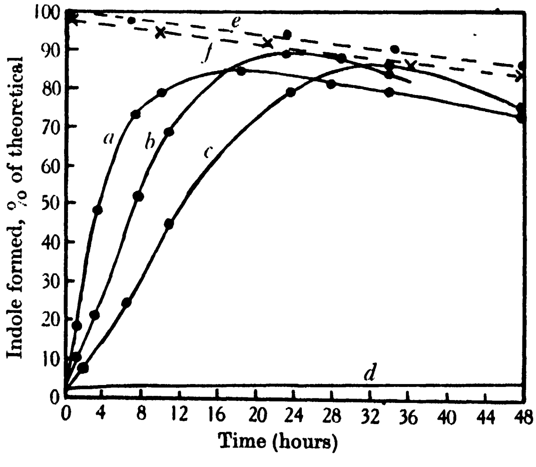

Fig. 1.

tryptophan solutions) was aerated together with 20 ml. of *Bact. coli* suspension (Fig. 1*e*). This curve gives the rate of loss of indole due to aeration. An experiment in which indole was aerated with a killed suspension of *Bact. coli* (Fig. 1*f*) proves that this loss is due to aeration and not to breakdown of tryptophan by *Bact. coli*, for the rate of loss of indole is identical in the two cases.

In Fig. 2 the curves *b* and *c* of Fig. 1 have been corrected for the steady loss of indole shown above and further for the average 4 % loss occurring in the distillation process. In each case the indole formed is shown to be 100 % of that theoretically obtainable from the tryptophan added. Four other experiments gave similar results.

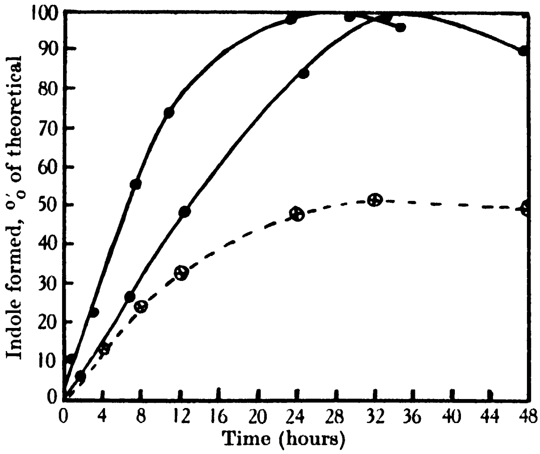

Fig. 2. ——— *l*-Tryptophan; - - - - *dl*-tryptophan.

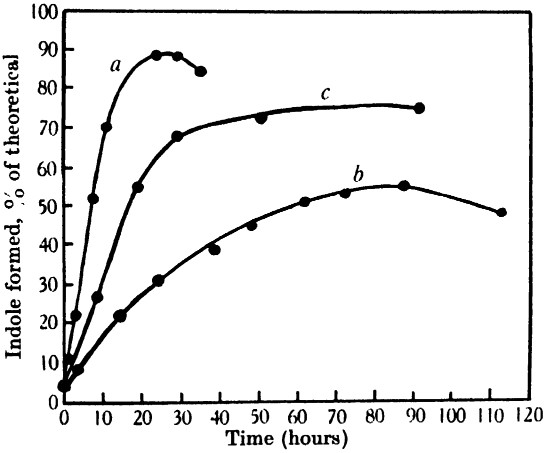

Fig. 3.

Hopkins and Cole [1903] isolated indoleacetic acid, as well as indole, from the products of the aerobic growth of *Bact. coli* on a medium of tryptophan, salts and gelatin; it will be seen that in the experiments described above there was no formation of this acid. This distinction is probably due to the different conditions under which the experiments were performed, for the present work using washed suspension differed from that of Hopkins and Cole in that: (*a*) the cultures were aerated, (*b*) a small amount of tryptophan and a large quantity of *Bact. coli* were used and (*c*) there were no other substrates present. It is possible that the presence of alternative sources of carbon and nitrogen modifies the course of the reaction. The course of breakdown of tryptophan by *Bact. coli* has been shown to be modified considerably by the addition of glucose and other sugars [Hertzfeldt and Klinger, 1915].

Effect of aeration. The degree of aeration was found to have a marked effect on the course and completeness of the reaction. In Fig. 3, curve *a* represents the (uncorrected) production of indole when air was continuously passed through the apparatus; curve *b* is from a non-aerated experiment; and curve *c* is from an experiment in which the culture was contained in a 500 ml. Ehlenmeyer flask. Attempts to detect indoleacetic acid in the culture filtrates at the end of the experiments were unsuccessful.

Rate of breakdown of tryptophan.

It was hoped that by studying the disappearance of tryptophan concurrently with the formation of indole further light might be thrown on the course of the reaction. In the previous work of Hertzfeldt and Klinger [1915] these had been shown to be roughly in agreement.

Two methods of estimating tryptophan were used and estimations of indole were carried out at the same time in the usual way.

(1) A colorimetric method making use of the condensation reaction between tryptophan and *p*-dimethylaminobenzaldehyde in the presence of hydrochloric acid. (Details as to quantities are from a private communication from Dr R. S. Alcock.) An aeration experiment with tryptophan solution and *Bact. coli* suspension was performed as described above and 10 ml. samples were removed for the estimation of indole and 15 ml. samples for the tryptophan determinations. The latter were filtered through kieselguhr and 12 ml. of the filtrate precipitated with 2 ml. of 30 % trichloroacetic acid and filtered with kieselguhr to remove proteins. 10 ml. of the filtrate were extracted three times with ether to remove indole and freed from ether by aeration. 4 ml. or less (according to the concentration of tryptophan, which should be 0·2–0·5 mg. in not more than 4 ml.) were taken for estimation. The tryptophan standard was diluted to the same volume as the test solution. To each were added 4 drops of 4 % *p*-dimethylaminobenzaldehyde (in alcohol) and 15 ml. of concentrated hydrochloric acid. After standing 2–3 days in the dark to develop the maximum blue colour, the solutions were diluted to 25 ml. and compared colorimetrically with a standard prepared in a similar way. In the later stages when the concentration of tryptophan had become very low, 6 and 8 ml. of the indole-free solution were taken for estimation and fuming hydrochloric acid was used instead of concentrated.

A typical result is shown in Fig. 4 (*a* and *b*). The broken curve (*a*) represents the percentage of tryptophan oxidised, the full line (*b*) the percentage of indole formed (corrected). It will be seen that there is complete correspondence.

(2) As tryptophan was the only amino-acid present it was possible to estimate it by determinations of amino-nitrogen. The manometric method of Van Slyke [Peters and Van Slyke, 1932] was chosen.

15 ml. samples from a tryptophan-*Bact. coli* aeration experiment were deproteinised by the addition of 4 ml. of 30 % trichloroacetic acid and filtered with kieselguhr. To 10 ml. of the filtrate were added 4–5 drops excess of Phillips's "milk of magnesia" and the solution was boiled down to half-volume in order to remove ammonia. After the addition of a slight excess of glacial acetic acid, the

clear solution was diluted to 10 ml. and 4 or 5 ml. samples were taken for estimation. A blank experiment was performed without tryptophan in order to determine the endogenous production of amino-N by *Bact. coli* from its own protein. The blank values were subtracted from the values obtained in the tryptophan experiment and the difference gives the amount of amino-N due to the tryptophan.

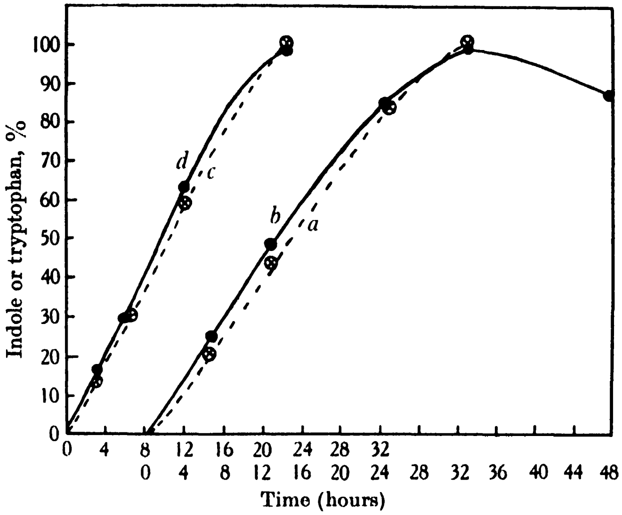

Fig. 4. ——— Indole formed, %; - - - - tryptophan broken down, %.

Table II gives the results of such an experiment.

Table II.

Time hrs.	Tryptophan exp. amino-N mg./100 ml.	Blank exp. amino-N mg./100 ml.	Amino-N due to tryptophan mg./100 ml.	% tryptophan remaining	% indole formed (corrected)
0	1·146	0·387	0·776	100	0
3·0	1·310	0·637	0·673	86·7	17·1
6·3	1·280	0·743	0·537	69·2	30·8
12·0	1·170	0·840	0·330	41·0	64·0
22·5	0·914	0·933	—	0	99·3

The results are given in graphical form in Fig. 4, curves *c* and *d*. It will be seen that identical results are obtained by the two methods; the rate of formation of indole is equal to the rate of disappearance of tryptophan and these experiments do not indicate any accumulation of intermediate products. The second method also measures the rate of deamination of tryptophan and the results lead to the conclusion that if more than one step is involved in the breakdown of tryptophan to indole, then the whole process is limited by the rate of deamination, since this is identical with the rate of formation of indole.

Oxygen uptake of the reaction.

The oxygen uptake of the oxidation of tryptophan to indole was measured in Barcroft manometers. 1 ml. *Bact. coli* suspension was placed in both cups; the left cup also contained 1 ml. buffer p_H 7·2 and 1 ml. water, and the right cup 1 ml. buffer and 1 ml. tryptophan solution (containing 1–5 mg. of tryptophan). The centre tubes of both cups contained 40 % KOH for the absorption of carbon dioxide. The reaction proceeded very slowly, however, and after 12 hours' shaking only 40 % of the theoretical amount of indole had been formed. Attempts were made to accelerate the reaction by adjustment of the concentration of the reactants, and also by the addition of oxygen carriers such as methylene blue, but with no success.

It was decided to put up a series of experiments and to compare the oxygen uptake with the indole formed at different periods. The contents of the Barcroft cups were washed out and diluted to a suitable volume and the indole was estimated in the usual way (see Fig. 5). The straight line relationship was more marked than was expected, for the high endogenous oxygen uptake of the thick *Bact. coli* suspensions necessary to bring about the reaction at a reasonable rate render small errors in the volume of suspension added very serious. The period of shaking (up to 12 hours) was also much longer than that normally used in accurate manometer work. The straight line relationship between oxygen uptake and indole formation renders justifiable a calculation of the oxygen uptake for the complete reaction. A series of eight experiments gave an average oxygen uptake of 472 μl. of O_2 per mg. of indole formed (Table III).

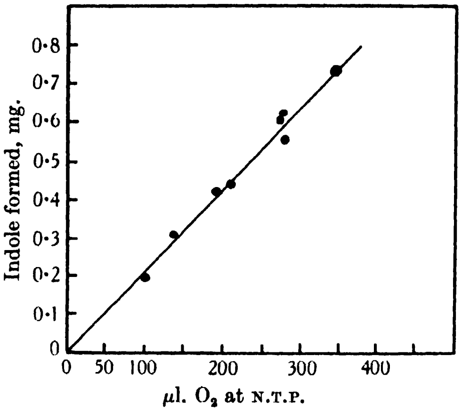

Fig. 5.

Table III.

O_2 uptake (μl. of O_2 at N.T.P.)	Indole formed mg.	μl. O_2 per mg. of indole	O_2 uptake (μl. of O_2 at N.T.P.)	Indole formed mg.	μl. O_2 per mg. of indole
101	0·191	528	278	0·608	457
134	0·312	428	279	0·628	444
190	0·418	455	281	0·543	517
207	0·432	479	345	0·735	469
				Mean	472

Theoretically, if the oxidation of 1 molecule of tryptophan is brought about by:

(*a*) 6 atoms of oxygen, then 570 μl. of O_2 per mg. of indole are required;
(*b*) 5 ,, ,, 475 ,, ,, ,,
(*c*) 4 ,, ,, 380 ,, ,, ,,

The conclusion seems unavoidable that 5 atoms of oxygen are involved in the oxidation of each tryptophan molecule. This agrees with an equation postulating the final complete oxidation of the tryptophan side chain to carbon dioxide and water, together with the elimination of nitrogen as ammonia:

$$C_8H_6N.CH_2CHNH_2COOH + 5O \rightarrow C_8H_7N + 3CO_2 + NH_3 + H_2O.$$

Experiments with inhibitors.

The effect of enzymic inhibitors on the course of the oxidation of tryptophan was studied. Barcroft manometric experiments were used for this purpose, since any change or stoppage of the reaction would be reflected by a change in the oxygen uptake. The following treatments were used:

(1) *Toluene.* The bacterial suspension was shaken with toluene, incubated for 10 min. at 37° and freed from toluene by aeration. The normal oxygen uptake was completely inhibited.

(2) *Chloroform.* The treatment was the same as with toluene, and in Barcroft experiments there was again no oxygen uptake.

(3) *Acetone. Bact. coli* suspension was centrifuged down, rubbed up with acetone, washed twice by centrifuging and finally suspended in the original volume of Ringer's solution. There was no oxygen uptake after this treatment.

(4) *Cyclohexanol.* The bacterial suspension was made 2 % with respect to cyclohexanol, incubated at 37° for 8–9 hours and washed as in (3). Oxygen uptake was again totally inhibited.

These results show the reaction to be very sensitive to inhibitors, and no information was obtained from them as to the course of the breakdown of tryptophan.

Anaerobic experiments.

A series of 75 ml. Thunberg tubes each containing 20 ml. of tryptophan solution of the usual strength and 4 ml. of *Bact. coli* suspension was set up. The tubes were evacuated, filled with nitrogen and incubated at 37°. At intervals during 48 hours tubes were taken out and indole was estimated. A regular formation of a small amount of indole, amounting in 48 hours to only 5·8 % of that theoretically possible, was noted. This is probably due to the presence of endogenous hydrogen acceptors in the bacterial suspension.

Hopkins and Cole [1903] have isolated β-indolepropionic acid from the products of the anaerobic growth of the Rauschbrand bacillus on tryptophan-gelatin medium. It was found impossible to devise a satisfactory method for the quantitative estimation of indolepropionic acid, but its anaerobic formation by *Bact. coli* suspensions from tryptophan was confirmed in the following way. After 48 hours' incubation the culture was filtered through kieselguhr, proteins were precipitated by warming at p_H 3·8 and the liquid was again filtered through kieselguhr. The filtrate was adjusted to p_H 1 and extracted with ether. The aqueous layer gave negative tests for tryptophan with bromine water and with the formaldehyde test. The ether solution, after concentration, was extracted twice with 1 % sodium carbonate solution and the extracts were diluted to the original volume of the tryptophan solution. Tests for β-indolepropionic acid were made on this solution by colour reactions given in detail in Table I of the following paper [Woods, 1935, p. 651]. A solution of β-indolepropionic acid of strength equivalent to the original tryptophan solution gave identical reactions of approximately the same intensity. No trace of any other indole derivative could be detected.

These results show that tryptophan is completely broken down by suspensions of *Bact. coli* under anaerobic conditions, and that the production of indolepropionic acid is almost quantitative.

Experiments with racemic tryptophan.

A sample of racemic tryptophan was prepared from *l*-tryptophan by the method given by du Vigneaud and Sealock [1932]. The production of indole from racemic tryptophan was studied by aeration experiments with *Bact. coli* suspension, similar to those already described for *l*-tryptophan.

A maximum production of 45·2 % of the theoretical quantity of indole was found. Correction for indole losses (see p. 643) brought this figure up to 51·0 %. The result of an experiment is shown in the broken curve of Fig. 2. The rate of fall of indole after the maximum was slower than usual, suggesting that there is a very slight and slow breakdown of the *d*-tryptophan. These experiments show that if *Bact. coli* is able to bring about the formation of indole from *d*-tryptophan at all, then it does so at a rate incomparably slower than from *l*-tryptophan.

SUMMARY.

1. The formation of indole from *l*-tryptophan by thick washed suspensions of *Bact. coli* has been studied. Under conditions of continuous aeration there is quantitative production of indole.

2. The degree of aeration has a marked effect on the rate and completeness of the reaction.

3. The rate of disappearance of tryptophan is identical with the rate of formation of indole.

4. The oxidation of one molecule of tryptophan to indole by *Bact. coli* requires five atoms of oxygen.

5. *d*-Tryptophan is broken down, if at all, at a very slow rate.

6. Under anaerobic conditions β-indolepropionic acid is formed.

My thanks are due to Miss M. Stephenson for constant advice and criticism, and to Sir F. G. Hopkins for his interest and encouragement.

REFERENCES.

Bergeim (1917). *J. Biol. Chem.* **32**, 17.
Berthelot (1914). *Ann. Inst. Pasteur.* **28**, 849.
Dakin (1918). *Biochem. J.* **12**, 302.
Fellers and Clough (1925). *J. Bact.* **10**, 105.
Herter and Foster (1905). *J. Biol. Chem.* **1**, 257.
Hertzfeldt and Klinger (1915). *Zentr. Bakt.* I (orig.), **76**, 1.
Hopkins and Cole (1901). *J. Physiol.* **27**, 418.
—— —— (1903). *J. Physiol.* **29**, 451.
Kilborn, Pierce and Tittsler (1928). *Proc. Soc. Exp. Biol. Med.* **26**, 391.
Kuhne (1875). *Ber. deutsch. chem. Ges.* **8**, 206.
Peters and Van Slyke (1932). Quantitative clinical chemistry, methods, 397. (London, Baillière, Tindall and Cox.)
Raistrick and Clarke (1921). *Biochem. J.* **15**, 76.
du Vigneaud and Sealock (1932). *J. Biol. Chem.* **96**, 511.
Woods (1935). *Biochem. J.* **29**, 649.
Zoller (1920). *J. Biol. Chem.* **41**, 37.

LXXVII. INDOLE FORMATION BY *BACTERIUM COLI.*

II. THE ACTION OF WASHED SUSPENSIONS OF *BACTERIUM COLI* ON INDOLE DERIVATIVES.

By DONALD DEVEREUX WOODS.

From the Biochemical Laboratory, Cambridge.

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A convenient technique for the quantitative investigation of indole formation having been described in the preceding paper [Woods, 1935], it was decided to study the action of thick washed suspensions of *Bact. coli* on various indole derivatives. Such derivatives, if intermediate stages in the breakdown of tryptophan by *Bact. coli*, should give rise to indole at least to the same extent and as rapidly as tryptophan itself under the same conditions.

Frieber [1922], in a series of growth experiments with *Bact. coli*, substituted a number of indole derivatives for tryptophan in his growth medium. He found no production of indole from the following compounds: α-indolecarboxylic acid, αβ-indoledicarboxylic acid, skatole, β-indolealdehyde, β-indoleacetic acid, β-indolepyruvic acid, β-indole-ethylamine. Saito [1933] found that, in growth experiments, *dl*-β-indolelactic acid (but not *l*-indolelactic acid) gave rise to indole when incubated in a suitable medium with *Bact. coli*. This formation of indole took place only in the presence of an alternative source of nitrogen—a mixture of ammonium lactate and asparagine was used. Unfortunately the tests for indole were qualitative only, and no data as to concentration of either indolelactic acid or indole are given, so that it is difficult to assess the importance of this result. Saito also finds that with β-indolepyruvic acid there is formation of indole if fresh cultures of *Bact. coli* are used. This was presumably in the presence of alternative sources of nitrogen, though this is not definitely stated; results were again qualitative. Saito's result with indolepyruvic acid does not agree with that of Frieber, whose medium also contained ammonium lactate, though not asparagine.

Preparation of the indole derivatives.

β-Indolepropionic acid was prepared from adipic acid by a series of reactions quoted by Berg *et al.* [1929]. The final colourless crystalline product melted at 133° (uncorrected). N (micro-Dumas), 7·35 %. Theoretical, 7·41 %. Equivalent (by titration in alcoholic solution), 188. Theoretical, 189.

β-Indolealdehyde was prepared as described by Majima and Kotaki [1922, 1], modified by Putochin [1926]. It was found more expedient to start the Grignard reaction in ether solution and to add the benzene solutions gradually. A considerable loss occurs during the steam-distillation of the crude product owing to resinification. This was avoided in the following way. The crude aldehyde formed on hydrolysis of the Grignard compound was centrifuged down, sucked dry on a Büchner funnel and washed on the funnel with a small amount of pure benzene. After drying at 37° it was ground up twice with light petroleum, filtered and dried. This treatment removes *N*-formylindole, indole and other unchanged reactants. The product was finally recrystallised twice from boiling water. From 10 g. of indole 3 g. of faint gold-coloured crystals M.P. 194° were obtained. N (micro-Dumas), 9·60 %. Theoretical, 9·66 %.

β-Indoleacetic acid was made from indole according to Majima and Hoshino [1925]. The chloroacetonitrile used was prepared from chloroacetamide [Steinkopf, 1908]. The product decomposed at 160–165° to skatole and carbon dioxide. N (micro-Dumas), 8·05 %. Theoretical, 8·00 %. Equivalent (by titration in alcoholic solution), 173. Theoretical, 175.

β-Indolecarboxylic acid. The method of Majima and Kotake [1922, 2] was used. The final product was faintly pink and melted at 215° (uncorrected). N (micro-Dumas), 8·64 %. Theoretical, 8·70 %.

β-Indoleacrylic acid was synthesised from β-indolealdehyde by the procedure described by Bauguess and Berg [1934]. The yellow crystals obtained melted with decomposition at 194° (uncorrected). N (micro-Dumas), 7·56 %. Theoretical, 7·49 %. Equivalent (by titration in alcoholic solution), 186. Theoretical, 187.

β-Indolepyruvic acid was prepared according to Ellinger and Matsuoka [1920] from β-indolealdehyde. The product, after removal of acetic acid of crystallisation, was buff-coloured. It darkened at 200°, softened at 206° and melted at 211° (all uncorrected). Berg *et al.* [1929] give these figures as 198°, 204° and 212°. N (micro-Dumas), 9·56 %. Theoretical, 9·66 %.

Experiments with indole derivatives.

In the work to be described the conditions were identical with those described in the preceding paper [Woods, 1935] in studying the breakdown of tryptophan. The following quantities were used: 100 ml. of a solution of the indole derivative in phosphate buffer p_H 7·2 (containing the chemical equivalent of 20 mg. of tryptophan). 20 ml. of *Bact. coli* suspension (prepared as described in the last paper). It was necessary to dissolve the derivatives in a small amount of dilute alkali as they are all soluble with difficulty in cold water. The p_H was adjusted to 7·2, buffer was added and the solutions were diluted to the required volume. They were sterilised by filtration and all experiments were conducted at 37°.

The culture filtrates used for testing for the derivatives were prepared in the following way. The suspension was filtered through kieselguhr and proteins were precipitated by warming at p_H 3·8. After filtering through kieselguhr the clear solution was adjusted to p_H 1–2 and extracted well with ether. The ether solutions were concentrated, extracted twice with 2 % sodium carbonate solution and the extracts diluted to the original volume of the suspension.

The colour reactions used for the detection of the various indole derivatives are given in detail in Table I.

Anaerobic experiments were conducted in Thunberg tubes, which were evacuated and filled with nitrogen.

β-Indolealdehyde. An aeration experiment with *Bact. coli* suspension was performed as described in the last paper. In 48 hours there was only the normal 1–2 % autolytic production of indole from the suspension itself.

β-Indolecarboxylic acid. There was no formation of indole from this derivative when aerated together with *Bact. coli* suspension.

β-Indoleacetic acid. This is a particularly interesting compound, since Hopkins and Cole [1903] have isolated it from the products of the aerobic growth of *Bact. coli* on tryptophan, whilst the washed suspension technique has been shown [Woods, 1935] to give only indole (in theoretical yield) and no trace of β-indole-

Table I.

All tests are on solutions containing 10 mg. of indole (or their equivalent) in 100 ml.

Test (3 ml. solution)	β-Indolepyruvic acid	β-Indoleacrylic acid	β-Indoleacetic acid	β-Indolepropionic acid	Skatole	Indole
1 ml. 50 % H_2SO_4 and 1–2 drops 2 % KNO_2	Purplish red colour insoluble in $CHCl_3$, soluble in amyl alcohol, giving cherry-red solution with absorption band at 550 $m\mu$ (1)	Yellow, then light pink colour, soluble in amyl alcohol to a pink solution	Cherry-red colour soluble in amyl alcohol	Golden yellow intensifying on warming. With higher concentrations crystalline needles on cooling. Soluble in amyl alcohol	Yellow in the cold, more orange on boiling. Soluble in amyl alcohol and in $CHCl_3$	Cherry-red colour, soluble in amyl alcohol, insoluble in $CHCl_3$
3 drops 2 % *p*-dimethylaminobenzaldehyde (in alcohol) and 1 ml. conc. HCl	No colour even on heating	No colour is given	Violet on boiling, deeper violet on adding 1 drop 2 % KNO_2. Insoluble in $CHCl_3$, soluble in amyl alcohol	Light rose, deepening on adding 1 drop 2 % KNO_2. Colour quickly goes orange and fades	Blue-violet colour on boiling, soluble in amyl alcohol and $CHCl_3$ to a pure blue solution. Addition of 1 drop 2 % KNO_2 gives intense blue colour, soluble in amyl alcohol and in $CHCl_3$	Red-violet colour soluble in amyl alcohol and $CHCl_3$ to a red-violet solution. Addition of 1 drop 2 % KNO_2 gives a more orange tint, soluble as before
3 drops 2 % *p*-dimethylaminobenzaldehyde (in alcohol) and 3 ml. fuming HCl	Orange on boiling. On adding 1 drop 2 % KNO_2 colour goes greenish yellow (soluble in amyl alcohol)	Orange on boiling. On adding 1 drop 2 % KNO_2 colour goes orange-red (soluble in amyl alcohol)	Similar to preceding test (above)	Red-violet colour on warming. Addition of 1 drop 2 % KNO_2 gives a deep blue-violet colour soluble in $CHCl_3$ to a deep blue-violet solution	Similar to preceding test (above)	Similar to preceding test (above)
1 drop conc. HCl and a few drops of very dilute $FeCl_3$	On warming first violet-red, then orange at higher temperatures	Orange-brown on boiling	Red-violet on boiling. Soluble in amyl alcohol	Orange solution on warming	Orange-red on boiling, intensified by 1 drop 2 % KNO_2	Very pale yellow on boiling. 1 drop 2 % KNO_2 gives a cherry-red colour
2 drops 1 % vanillin (in alcohol) and 3 ml. conc. HCl	Orange colour on boiling. Addition of 1 drop 2 % KNO_2 gives colour as in (1)	Orange on boiling. Addition of 1 drop 2% KNO_2 gives a deeper colour	Pale pink on boiling, soluble in amyl alcohol. Colour intensifies on adding 1 drop 2 % KNO_2, soluble in amyl alcohol to a pink-violet solution	Light purplish pink on boiling, insoluble in $CHCl_3$. Addition of 1 drop 2 % KNO_2 gives a deep violet colour soluble in $CHCl_3$	Permanganate colour on boiling, soluble in amyl alcohol and $CHCl_3$ to a violet solution. 1 drop 2 % KNO_2 gives a deep violet colour soluble as before	Deep orange colour soluble in amyl alcohol, almost insoluble in $CHCl_3$. Addition of 1 drop 2 % KNO_2 causes colour to fade
Shaken with 1 drop dimethylaniline and 3 ml. conc. HCl added	Orange on boiling	Orange on boiling	Pink on boiling, insoluble in $CHCl_3$, soluble in amyl alcohol	Blue-violet colour on heating, soluble in $CHCl_3$ giving a purple-red extract	Violet colour on boiling, soluble in amyl alcohol and in $CHCl_3$	Pale orange pink on boiling, partly soluble in $CHCl_3$, soluble in amyl alcohol
50 ml. solution, 0·5 ml. 1 % Na β-naphthoquinonemonosulphonate and 1 ml. 10 % KOH					No colour is formed	Blue colour develops in 15 mins. (or precipitate with strong solutions), soluble in $CHCl_3$ to an orange-red solution

acetic acid. However, aeration experiments showed that, in 48 hours, this acid gave rise to no indole, beyond the normal 1–2 % blank, when incubated with *Bact. coli* suspensions. No trace of skatole was observed. A culture filtrate at the end of the experiment gave reactions identical with the original indoleacetic acid solution, and there was no trace of any other derivative.

Equal quantities of β-indoleacetic acid and tryptophan were mixed and an aeration experiment performed; a normal curve for the production of indole from tryptophan was obtained, showing that indoleacetic acid is neither toxic to *Bact. coli* nor inhibitory of the production of indole.

Anaerobic experiments were also done with β-indoleacetic acid and *Bact. coli* suspension in order to determine if skatole could be formed under these conditions. After 50 hours' incubation the culture distillates showed no trace of indole or skatole. Salkowski E. and H. [1885] have shown that the sodium salt of indoleacetic acid when sown with a mixed putrefactive culture gave no indole or skatole.

β-Indolepropionic acid. This is the end-product of the anaerobic action of *Bact. coli* on tryptophan. Aeration experiments showed that it was not broken down to indole under aerobic conditions, as in 48 hours only the usual small blank was obtained. When mixed with tryptophan it did not inhibit the normal production of indole from this amino-acid. A culture filtrate was prepared at the end of the experiment; it gave tests identical with a solution of pure indolepropionic acid and there was no trace of indoleacetic acid or of any other derivative. These experiments show that the product of the anaerobic breakdown of tryptophan is quite unchanged when incubated with *Bact. coli* aerobically.

β-Indolepyruvic acid. In view of the oxidative nature of the breakdown of tryptophan to indole, it was thought possible that an initial stage might be an oxidative deamination to β-indolepyruvic acid. Some results by other workers with this derivative have already been mentioned. Aeration experiments were performed using freshly made suspensions of *Bact. coli*, but only the normal blank of 2 % indole was obtained in duplicate experiments (Fig. 1, curve *a*). Tests of the culture filtrate indicated that the acid was unchanged, and in addition identical derivatives with 2:4-dinitrophenylhydrazine were obtained from the culture filtrate and from a solution of pure indolepyruvic acid.

The usual experiment to test the possibility of the derivative itself inhibiting the formation of indole led to a surprising result. When a solution containing both indolepyruvic acid and tryptophan was aerated in the presence of *Bact. coli* suspension, 10–15 % more indole was formed than could have been derived from the tryptophan present. This is shown in curve *b* of Fig. 1. Curve *c* shows the formation of indole from the same weight of tryptophan as was used for *b*. The excess formation of indole is outside the limits of experimental error (see previous communication, Table I). Bearing in mind that Saito's [1933] experiments—in which indole was obtained from β-indolepyruvic acid—were conducted in the presence of an alternative source of nitrogen, it seemed of interest to try the effect of replacing tryptophan by other nitrogen compounds. Ammonia was chosen as the simplest possible.

In one experiment 30 mg. of ammonium sulphate were added to 20 mg. of indolepyruvic acid in 100 ml. of solution; in another the indolepyruvic acid was dissolved in dilute aqueous ammonia and the p_H adjusted to 7·4 (the ammonia present was equivalent to 300 mg. of ammonium sulphate). The results of aeration experiments with these solutions are shown in Fig. 1, curves *d* and *e*. It is seen that indole was formed in appreciable quantity (8–10 %) in the first 8–10 hours and then remained steady for 20 hours, after which there was a slight fall.

Since indole is continually lost by aeration (see previous paper, p. 650), the constant period is really a period of slightly increasing indole concentration reaching 1–2 % above the maximum recorded. The experiments are good duplicates, showing that increasing the concentration of ammonia has no effect. Further, the addition of more ammonium sulphate during the steady period did not increase the yield of indole.

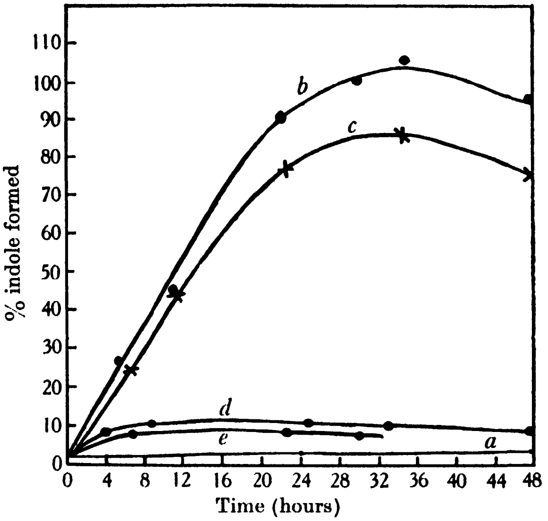

Fig. 1.

The effect of tryptophan in the experiment shown in curve *b* is probably due, therefore, to ammonia formed from the deamination of tryptophan during its breakdown to indole, for the amount of extra indole is approximately equal to the indole obtained in the presence of ammonia. The possible significance of these results will be discussed later.

The action of *Bact. coli* on β-indolepyruvic acid under anaerobic conditions was also tried. The usual proportions of substrate and suspension were incubated anaerobically for 48 hours and a protein-free filtrate prepared. On testing the filtrate there was no trace of β-indolepropionic acid and the indolepyruvic acid was unchanged.

β-Indoleacrylic acid. When an aeration experiment with *Bact. coli* was carried out there was no formation of indole beyond the normal 2 % blank. The possible inhibitory action of β-indoleacrylic acid on indole production was tested and a normal formation of indole from tryptophan was found. The addition of ammonium sulphate did not bring about any formation of indole, and in all cases tests on the culture filtrate showed the acid to be unchanged, no trace of any other indole compound being detectable.

Anaerobic experiments were also done:

(*a*) With indoleacrylic acid and *Bact. coli* suspension alone.

(*b*) With indoleacrylic acid and suspension in the presence of formate as a hydrogen donator (10 mg. sodium formate to 20 ml. of solution).

(*c*) With indoleacrylic acid and suspension in the presence of both formate and ammonium sulphate (10 mg. of each to 20 ml. of solution).

The culture filtrate after 50 hours' incubation showed no formation of indolepropionic acid in any of the experiments, and the indoleacrylic acid was unchanged.

DISCUSSION.

The growth experiments of Frieber [1922] were repeated in a number of cases (β-indolealdehyde, β-indoleacetic acid and β-indolepyruvic acid) by the washed suspension technique and his results were confirmed. It seems probable, therefore, that his other experiments, of which β-indole-ethylamine is of most physiological importance, would also be confirmed. This duplication of results provides support for the validity of the use of washed suspensions. Work described in the previous paper shows that a theoretical yield of indole can be obtained from tryptophan by the action of washed suspensions of *Bact. coli*; there seems little reason to doubt that, if any of the indole compounds used had been intermediates in the breakdown of tryptophan to indole, then they would have given rise to indole in something like quantitative yield, especially as in all the cases tried the derivatives themselves were not inhibitory of the normal production of indole from tryptophan.

The only case in which some indole was obtained—that of β-indolepyruvic acid—is of peculiar interest. In growth experiments, such as those of Saito [1933], it is reasonable that an alternative source of nitrogen should be necessary, since nitrogen is essential for growth of the organism and *Bact. coli* is not able to split the indole ring to obtain this nitrogen. But that an alternative source of nitrogen should be necessary for the formation of indole from indolepyruvic acid in washed suspension experiments must cast doubt on any suggestion that β-indolepyruvic acid is an intermediate, quite apart from the fact that only 8 % of the indole theoretically possible is obtained, compared with 100 % from tryptophan. A possible explanation of this formation, and also of the formation of indole from *dl*-β-indolelactic acid noted by Saito [1933], is that there is some synthesis of tryptophan from indolepyruvic acid and ammonia, and that this tryptophan is then oxidised in the usual way to indole.

It has been suggested, Harden [1930], that the bacterial decomposition of tryptophan occurs by the following route: tryptophan → β-indolepropionic acid → β-indoleacetic acid → indole. The work here reported gives no support to this hypothesis.

In the breakdown of tryptophan to indole, decarboxylation may either precede or follow deamination. In the former case β-indole-ethylamine should result, but Frieber's [1922] work showing that this amine does not give rise to indole with *Bact. coli* is evidence that this does not occur. In the latter case an oxidative deamination to β-indolepyruvic acid seems probable in view of the aerobic nature of the reaction, but, as has been already pointed out, experiment does not support this theory.

Raistrick [quoted by Cole, 1933] has suggested that the primary reaction in both aerobic and anaerobic breakdown of amino-acids by bacteria is a deamination to the unsaturated acrylic acid. This suggestion is based on the isolation of unsaturated acids from the corresponding amino-acids (histidine, tyrosine and aspartic acid) after bacterial action. Indoleacrylic acid has not, so far as the author knows, been reported as having been obtained in this way. The experiments which have been described show that β-indoleacrylic acid does not give rise either to indole aerobically or to indolepropionic acid anaerobically. Raistrick further suggests that the breakdown may proceed as follows:

$$R.CH_2.CH(NH_2).COOH \rightarrow R.CH{:}CH.COOH \rightarrow R.C(OH){:}CH.COOH \rightleftarrows$$
$$R.CO.CH_2COOH \rightarrow R.COOH \rightarrow RH.$$

This avoids β-indolepyruvic acid, but postulates instead a β-keto-acid as an intermediate product. This β-substituted compound would be very unstable and difficult to prepare in a free form, so it was not possible to test this experimentally. Since indole was produced from neither β-indoleacrylic acid nor β-indolecarboxylic acid, it seems unlikely that this series of reactions actually occurs. Indolecarboxylic acid has been detected by Berthelot [1914] in 15-day cultures of *B. proteus* on tryptophan-gelatin medium, but there is no justification for generalising from one bacterial species to another, and in any event the acid is probably a stable end-product formed under the given conditions.

It has been shown in the preceding paper that 5 atoms of oxygen are involved in the oxidation of the tryptophan molecule to indole, and that the side chain is completely oxidised to carbon dioxide, water and ammonia. It was also demonstrated that the rate of indole formation is limited by the rate of deamination of the tryptophan, but the results given in the present paper do not suggest any scheme for the course of the reaction. It is possible, as in all metabolic work, that the intermediate compounds are highly unstable substances of short lifetime which are passed on immediately from one enzyme to the next. The formation of indoleacetic acid as well as indole when the conditions are different [Hopkins and Cole, 1903] suggests that a series of reactions does occur, β-indoleacetic acid being the stable end-product of a branch from the main course of the action; it is improbable that indole is formed by a direct splitting off of the side chain of tryptophan from the indole nucleus.

SUMMARY.

1. The action of washed suspensions of *Bact. coli* on certain indole derivatives has been investigated.

2. There was no formation of indole from the following compounds: β-indolealdehyde, β-indolecarboxylic acid, β-indoleacetic acid, β-indolepropionic acid and β-indoleacrylic acid.

3. β-Indolepyruvic acid, but only in the presence of ammonia, gives rise to a 10 % production of indole.

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REFERENCES.

Bauguess and Berg (1934). *J. Biol. Chem.* **104**, 675.
Berg, Rose and Marvel (1929). *J. Biol. Chem.* **85**, 219.
Berthelot (1914). *Ann. Inst. Pasteur*, **28**, 849.
Cole (1933). Practical physiological chemistry, 240. (W. Heffer and Sons, Cambridge.)
Ellinger and Matsuoka (1920). *Z. physiol. Chem.* **109**, 259.
Frieber (1922). *Zentr. Bakt.* I (orig.), **87**, 254.
Harden (1930). Med. Res. Counc. System of Bacteriology, I, 242.
Hopkins and Cole (1903). *J. Physiol.* **29**, 451.
Majima and Hoshino (1925). *Ber. deutsch. chem. Ges.* **58**, 2043.
—— and Kotake (1922, 1). *Ber. deutsch. chem. Ges.* **55**, 3859.
—— —— (1922, 2). *Ber. deutsch. chem. Ges.* **55**, 3865.
Putochin (1926). *Ber. deutsch. chem. Ges.* **59**, 1987.
Saito (1933). *Z. physiol. Chem.* **214**, 28.
Salkowski, E. and H. (1885). *Z. physiol. Chem.* **9**, 8.
Steinkopf (1908). *Ber. deutsch. chem. Ges.* **41**, 2541.
Woods (1935). *Biochem. J.* **29**, 640.

LXXVIII. THE PREPARATION OF SEMI-PERMEABLE SAC-MEMBRANES.

By JOHN DUCKWORTH.

Rowett Research Institute, Bucksburn, Aberdeen.

(*Received January 31st, 1935.*)

Prior to a series of studies on the partition of serum-calcium in the case of the lactating cow it was felt that a study of methods of preparing membranes and of their use in ultrafiltration would be advisable. A survey of the literature revealed a number of methods for the preparation of membranes but the time required for the manufacture and the manipulation of somewhat elaborate apparatus were definite obstacles. It was consequently felt that the introduction of a method which would give membranes capable of duplication by different workers and easy of preparation and manipulation would be advantageous.

Two contributions to membrane preparation which appear to have received little consideration are:

(1) Trendelenburg's method [1923] of casting membranes on the outside of test-tubes.

(2) Pierce's method [1927] of limiting the porosity of flat membranes by the use of ethylene glycol.

By combining these two methods the objective has been attained.

Reagents and apparatus.

7 °/₀ Collodion solution. B.D.H. collodion is analysed for nitrocellulose content and diluted with an alcohol-ether solution (1:1 by weight) until it contains 7 % by weight of nitrocellulose.

Membrane solution. To the 7 % nitrocellulose solution is added the requisite amount of ethylene glycol found to be satisfactory by experimental trial.

15 °/₀ gelatin solution in water.

Gies reagent [*1910*]. To 1 litre of 10 % KOH solution add 25 ml. of 3 % $CuSO_4$ solution.

Test-tubes. A small hole is blown in the tip of each test-tube. The size of test-tube required depends on the amount of serum available for analysis.

The ultrafiltration apparatus. It was felt that the elimination of automatic valves and compressed air cylinders would be advisable. Ultrafiltration is carried out at a positive pressure of 175–185 mm. of Hg. and the maintenance of this pressure for one hour, without attention, can be achieved by the use of the pressure unit herein described (Fig. 1).

A. Manometer to register 175–220 mm. Hg.

B. A tube containing a Bunsen valve. This valve is necessary since it is difficult to obtain a pressure bulb which does not allow some air-leakage.

C. A tube containing normal saline.

D. A Winchester bottle as a reservoir of compressed air.

E. A wide-mouthed bottle which connects the pressure unit with the individual ultrafiltration units. In our investigations four ultrafiltration units are connected to this bottle.

F. Serum reservoir.

G. Membrane.

H. Ultrafiltrate tube.

I. A wide-mouthed bottle containing physiological saline. Since the vapour pressures of normal saline and ultrafiltrates are practically identical the losses by evaporation are minimised.

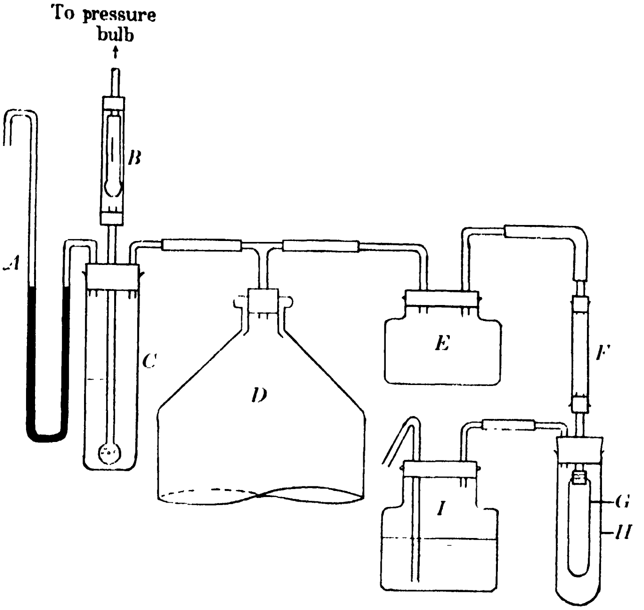

Fig. 1. Ultrafiltration apparatus.

Preparation of membranes. The test-tubes are coated with gelatin by dipping the stoppered tube into warm gelatin solution. The tubes are immersed to within 1 cm. of the lip, withdrawn and suspended in a special rack to drain and dry for several hours. The small hole in the tip should be sealed by a film of gelatin. Once dry these tubes can be stored until required.

To prepare the membrane the gelatined tube is dipped into the collodion-ethylene glycol solution to the required depth and slowly withdrawn. The tip of the tube is left in contact with the surface of the solution for a few seconds to facilitate draining and the tube is then rapidly transferred to the special rack and suspended. When removing the tube it is advisable to guard against drops of collodion falling into the solution, since this causes air-bubbles which will be picked up by subsequent tubes and cause faults in the membranes. The strand of nitrocellulose which forms at the tip of the tube as a result of draining is cut off as it forms, since the weight of it draws off too much nitrocellulose and results in fragile membranes. The entire preparation of the membrane should take about 20 seconds.

After the membranes have been drying in the rack for 20 minutes (exactly) the tube is filled with water and placed in a beaker of distilled water. The membrane should be entirely below the surface. After 1 hour or so the tubes are filled with warm water and shaken vigorously to dissolve the gelatin sealing the small hole at the tip of the tube and also to melt the gelatin film between the membrane and the glass. When the membrane slides free it is removed and filled with distilled water.

Instead of tying the membranes on to rubber stoppers it has been found more convenient to tie them on to pieces of rubber pressure tubing 1·5 cm. in length. When used in the ultrafiltration unit the glass tube connecting the membrane to the serum reservoir is somewhat larger than is generally used with this size of rubber tubing. The extra distension of the rubber is enough to cause the membrane to grip the tubing quite securely. This method was found to be so satisfactory that the connections of the serum reservoir are also made from rubber tubing instead of stoppers.

After being tied on to the rubber tubing the membrane is washed free from gelatin by shaking several times with distilled water. Finally the membrane is filled with distilled water and connected to the serum reservoir, which is then filled with distilled water, and 5 ml. of water are forced through the membrane at a pressure of 220 mm. of Hg. This removes the ethylene glycol and detects faulty membranes. It is advisable not to let the level of the water fall as low as the sac since the drying of any portion of the membrane will result in fragility at that point. When the membrane is removed it is filled to the top with distilled water and closed with a short glass rod. The membranes are stored in chloroform water until required.

Determination of ethylene glycol-nitrocellulose ratio. The proportion of ethylene glycol to nitrocellulose necessary to give a sac-membrane impermeable to serum proteins is slightly variable, depending on the sample of collodion.

To a series of tubes each containing a known amount of the 7 % nitrocellulose solution are added increasing amounts of ethylene glycol. The range employed is 1·5–2·0 ml. of ethylene glycol to 1 g. of nitrocellulose. Membranes are cast by the method described above and tested by ultrafiltering a sample of serum. Such membranes as show a negative biuret test when Gies reagent is added correspond to the maximum dilution permissible. It is advisable to keep somewhat below this.

So far it has been found that a proportion of 1·8 ml. of ethylene glycol to 1·0 g. nitrocellulose (*i.e.* 12·6 ml. of ethylene glycol to 100 ml. of the diluted collodion) gives uniformly biuret-negative filtrates.

Ultrafiltration method. When required for analytical purposes the membrane is rinsed out three times with serum, the washings being removed by means of a pipette, and filled with serum. The sac is then connected to the reservoir and the reservoir filled with serum. The pressure is raised to 180 mm. of Hg. and the outside of the sac dried with filter-paper. The ultrafiltrate appears almost immediately. After about 10 minutes the pressure is reduced to about 50 mm. (sufficient to keep the sac distended to facilitate handling) and the sac dried. The ultrafiltrate tube is then connected over the sac and the pressure raised to 185 mm. The pressure falls very gradually, usually to about 175 mm. in 1 hour.

RESULTS.

It is unnecessary to quote results regarding permeability. Sac-membranes made by this method were found to be completely permeable to aqueous solutions of $CaCl_2$ with a calcium content of 10 mg. per 100 ml.

The main point under investigation was that of duplication. Consequently three membranes each were made by four members of this Institute and used to prepare ultrafiltrates from a sample of serum obtained from slaughter-house blood. Each ultrafiltrate was analysed in triplicate. The results are shown in full in Table I.

Table I.

Results in mg. Ca per 100 ml. of ultrafiltrate.

Made by	Membrane number	Analysis 1	Analysis 2	Analysis 3	Average
A	1	6·0	6·0	6·1	6·0
	2	6·1	6·0	6·0	6·0
	3	5·9	6·1	6·0	6·0
B	1	5·8	5·9	5·8	5·8
	2	5·9	—	5·9	5·9
	3	5·9	6·0	5·9	5·9
C	1	5·8	5·9	5·9	5·9
	2	5·9	6·0	5·8	5·9
	3	6·0	5·9	—	6·0
D	1	6·0	5·9	6·0	6·0
	2	5·7	5·9	5·8	5·8
	3	5·8	5·9	6·0	5·9

The calcium estimations were by the method of Clark and Collip [1925]. The experimental error of this method is ± 2 %. A study of the results in Table I indicates that the variation in the calcium contents of the various ultrafiltrates was of this order. It would appear, therefore, that membranes made by the proposed method, and by different investigators, will yield identical results.

It is a point of interest that the membranes used in this experiment were two days old, with the exception of those prepared by D, which were over a week old. The results do not appear to be affected by the age of the membrane, at least within this limit. Also the membranes used were those prepared for the first time by all workers except D.

SUMMARY.

A method of preparing sac-membranes is described which will yield practically identical ultrafiltrates, at least in so far as the calcium is concerned, at the hands of different investigators.

REFERENCES.

Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.
Gies (1910). *J. Biol. Chem.* **7**, 60.
Pierce (1927). *J. Biol. Chem.* **75**, 795.
Trendelenburg (1923). *Pflüger's Arch.* **199**, 237.

LXXIX. THE ULTRACENTRIFUGAL PROTEIN SEDIMENTATION DIAGRAM OF NORMAL HUMAN, COW AND HORSE SERUM.

By ARTHUR SPROUL McFARLANE.[1]

From the Institute of Physical Chemistry, Upsala, Sweden.

(*Received February 1st, 1935.*)

It has been shown in a previous paper [McFarlane, 1935] that the diagrams which are obtained in the refractive index method of following protein sedimentation in the ultracentrifuge are capable of yielding a great deal of valuable qualitative and quantitative information relating to the composition of the protein solution in the centrifuge cell. In particular, they are capable of giving the concentration of each molecular fraction which separates in the centrifugal field and an estimate of the relative degree of dimensional homogeneity of the constituent molecules of each fraction. In the work to be reported here we have obtained the sedimentation diagrams for various sera and have attempted to establish the following general points: (1) the extent to which the ultracentrifugal sedimentation diagram is a strictly reproducible characteristic of a serum; (2) the nature and extent of any changes in the diagram caused by treating the serum in various ways; (3) whether the serum from a series of members of the same species gives the same sedimentation diagram; and (4) to what extent the serum diagram of normal members of different species is the same.

It is to be emphasised at the outset that the work which will be reported is essentially of the nature of a general survey of this new field of investigation, the object being more to discover the possibilities of the field as a whole than to establish any particular points in great detail.

Since each complete ultracentrifugal experiment takes some considerable time it has been necessary throughout to minimise the number of experiments. The evidence in regard to (1) is, therefore, largely obtained incidentally in the experiments designed to investigate (2) and (3). We have used cow serum in investigating the effect of treatment on the same sample of a serum because a large quantity was readily available to cover all the experiments. It was more convenient, and also of greater interest, to use human serum in investigating (3), and for the species comparison in (4) we have used human, cow and horse sera.

It is necessary to refer the reader to the previous paper [1935] for a full description of the theory and technique of an ultracentrifuge experiment and for the details of the methods of calculating the results. We shall require in the present communication to refer frequently to that paper.

SECTION 1. THE SEDIMENTATION DIAGRAM OF NORMAL COW SERUM.

In this section we shall describe a series of experiments all performed on the same sample of cow serum and designed to investigate the effect of treatment of various kinds on the sedimentation diagram of the serum.

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[1] Beit Memorial Research Fellow.

A large sample of blood was obtained from a cow which had no signs or history of disease.[1] The blood was allowed to clot spontaneously and the serum was separated after 12 hours. Portions of this serum were used in the following experiments. During the period of these experiments (10 weeks) the serum was kept at 4° under sterile conditions, and no signs of decomposition could be detected.

A portion of the serum was placed in a collodion dialysis bag arranged as for an osmotic pressure measurement. The bag was placed in 10 times its volume of 1 % sodium chloride solution and the level of serum in the capillary kept constant by externally applied pressure from a water manometer. When equilibrium had been reached, as indicated by constant manometer pressure, the apparatus was dismantled and the refractive index of solution and dialysate measured by means of the Pulfrich refractometer and using the sodium D line. The dialysate gave a negative protein reaction with salicyclsulphonic acid. By subtracting the two values we obtained the total protein refraction increment of the serum, subject to the assumptions regarding the effect of the diffusible ion concentrations discussed in the previous paper.

In calculating protein concentrations from our refractometric data we have used the following specific refraction increment values throughout this work:

Protein	Specific refraction increment	Author
Horse serum albumin	0·00183	Adair and Robinson [1930, 1]
Horse serum globulin	0·00186	Adair and Robinson [1930, 1]
Total horse serum	0·00184	—
Human serum albumin	0·00200	Schretter [1926]
Human serum globulin	0·00219	Schretter [1926]
Total human serum	0·00202	Schretter [1926]
Cow serum albumin Cow serum globulin Total cow serum	0·00185	—

The value for total horse serum protein is a mean of Adair and Robinson's values for horse serum albumin and globulin, and the cow serum values are assumed to be the same as the horse and sheep serum values which Adair and Robinson have given. The use of these values can only give approximate concentrations because of these assumptions, and because there is a good deal of difference of opinion in the literature as to the exact values of the specific refraction increment of the serum proteins, and particularly of human serum protein [*cf.* Linder *et al.*, 1924; Starlinger and Hartl, 1925; Schretter, 1926]. In this work we are primarily concerned to measure and compare differences in refractive index (n_1-n_0), concentration measurements being of secondary importance. We have, therefore, not made any more accurate determinations of concentrations than are given by the refractometric data, and we have avoided basing any important conclusions on the concentration values given.

In order to be able to compare the measured refractivity of the protein with the value calculated from the sedimentation diagram we have applied to the measured values the wave-length correction factor which Pedersen and Andersson, as described in our previous paper [1935], have obtained for horse serum proteins, *viz.* 1·08.

Exp. 1. Untreated cow serum. A portion of the serum was examined in a centrifuge cell 2·0 mm. thick. The centrifuge reached 58,000 r.p.m. (equivalent to a mean centrifugal force of 240,000 times gravity) after 40 mins. and was kept at this speed to within ± 300 r.p.m. throughout the run which lasted a further 3 hours and 3 mins. up to the time of the last exposure.

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[1] I am indebted to Med. Kand. O. Mellander for the supply of this serum.

It was found on viewing the scale from the camera when regular sedimentation had been established that an optical scale distance of 1·54 cm. gave satisfactory results, *i.e.* a large line displacement with good definition of the displaced lines, and this was used in all exposures. The temperature of the cell at the time of the first exposure (which we will call T_f) was 33·4°, and at the time of the last exposure (T_l) was 35·2°. The photographs were taken on Cramer plates using the mercury line at 366$m\mu$, which was isolated by means of a Corning nickel oxide glass filter. The exposure time was 25 secs., and the photographic enlargement factor 0·996.

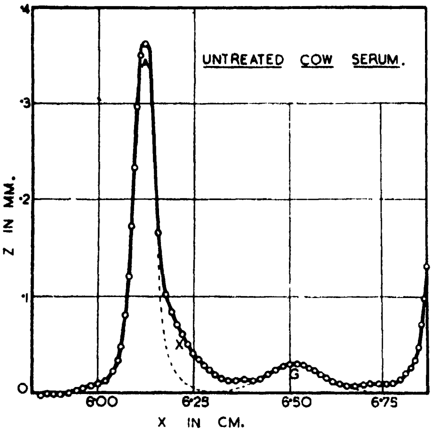

Fig. 1.

The curve shown in Fig. 1 was obtained from a photograph taken 108 mins. after reaching full speed. The position of the meniscus (not shown in the figure) is 5·64 cm. from the centre of rotation. It will be seen that three molecular fractions are present. $S_{W,20}$ for the lightest fraction is 2·67 and for the heaviest fraction is 4·60. These $S_{W,20}$ values are corrected for the density and viscosity of the electrolyte medium in which the protein is dissolved (*cf.* equation (5) of previous paper) but take no account of the concentration of the protein. The value of $S_{W,20}$ at infinite dilution (or the true sedimentation constant) of the *A* fraction may be obtained by extrapolating to infinite dilution a series of values of $S_{W,20}$ at various concentrations. It is not so easy to obtain the true sedimentation constant of the *X* or *G* fractions because it is necessary to take into account the effect of the concentration of the lighter fraction or fractions, as the case may be. In this work we are mainly concerned to identify a particular fraction, and for this purpose it is not necessary to obtain the extrapolated sedimentation constant. It is to be emphasised, however, that the $S_{W,20}$ values in this paper may not be compared with the true sedimentation constants published for other proteins, since these latter are all extrapolated and represent natural constants characteristic of the proteins and independent of the concentration. $S_{W,20}$ for the intermediate fraction cannot be obtained with any degree of accuracy because a clear separation of this curve from that of the lightest fraction was not obtained. We shall in future label the three fractions, in order of increasing sedimentation velocity, as *A* fraction (presuming a relationship with serum albumin); X fraction (of unknown nature); and G fraction (presuming a relationship with serum globulin).

It is impossible to produce here more than one curve from each experiment. This is a disadvantage because to obtain a complete picture of the sedimentation process it is necessary to consider all the curves.

We may discuss here in some detail a point of importance which arises in connection with the A and X fractions. We find in the case of cow serum the phenomenon of two boundaries which, in the process of sedimentation, reach the bottom of the cell without completely separating. The question arises whether under these conditions of incomplete separation we may attempt to resolve the composite curve graphically on the assumption that it consists of two symmetrical component curves. The problem is more comprehensible if we consider the boundaries in terms of protein concentration instead of refraction gradient. We have represented in Fig. 2A the refraction diagram of a hypothetical composite $A+X$ boundary with the corresponding protein concentration diagram shown in association with it (Fig. 2B). In both diagrams the component curves are represented by thin lines and the composite curve by a heavy line. Above the level "a" in the cell (*i.e.* to the left of the line "a" in the diagram) only A molecules are present, and we may therefore assume that the shape of this part of the curve will correspond to that of the sedimenting boundary curve of a pure solution of A molecules. As we have discussed in the previous paper, the concentration distribution of these molecules is determined by the forces of natural diffusion and an opposing force due to the variation of sedimentation velocity with concentration. Below the level "c" the shape of the curve should correspond to that of the boundary curve of a solution of X molecules sedimenting in a homogeneous medium of A molecules. Between

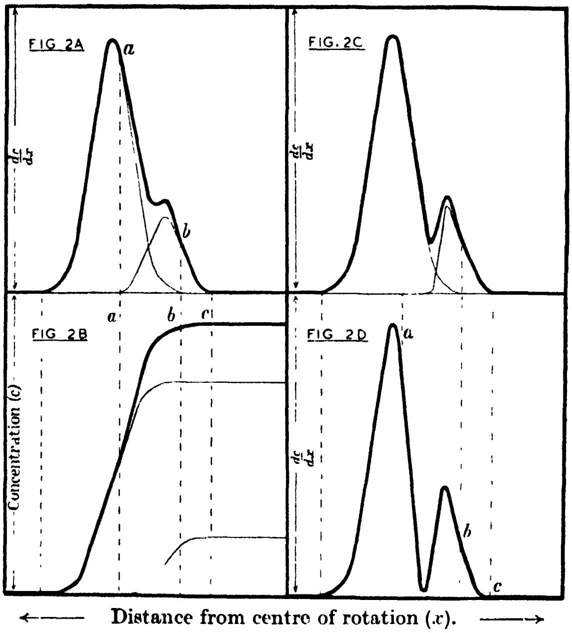

Fig. 2. Illustrating the type of curve-distortion caused by the overlapping boundaries in undiluted serum.

the levels "a" and "b' both molecular types are present in continuously varying concentration and it is difficult to visualise clearly the forces which will determine the ultimate shape of the composite concentration curve. It seems at least definite that in this region the X boundary gradient will be increased by the effect on the sedimentation velocity of the X molecules of the increasing concentration of A molecules as we proceed from "a" to "b". If this were the only factor present we should expect a composite curve of the type shown in Fig. 2c. Actually, however, we obtain curves of the general asymmetrical shape shown in Fig. 2D. The degree of distortion is not marked in the case of cow and horse sera but is characteristically pronounced in the case of untreated normal human sera. To explain the shape of this curve it is necessary to assume that in addition to the increase of the X concentration gradient there has also occurred an apparent "sharpening" of that part of the A boundary which lies between the levels "a" and "b", so that the limb of the curve nearer to the bottom of the cell comes to lie more vertically. It seems unlikely that this sharpening can be attributed to any effect of the concentration of X molecules on the sedimentation velocity of A molecules because the X molecules are sedimenting faster than the A molecules. It is possible, however,, that the diffusion of A molecules in this region will be influenced by the changing viscous effect of the solution of X molecules. The question of the influence of the charge on the molecules also arises, but a significant charge effect is regarded as unlikely in the presence of approximately 1 % neutral electrolyte. The possibility may not be excluded, however. It is not possible to obtain a more exact idea of the phenomenon until we have more data regarding the behaviour of the individual components of a mixture under the conditions of a simple diffusion experiment. We may note, however, that since the asymmetry shown in Fig. 2D is probably due to an external mechanical influence it should not affect the total area enclosed by the curve and the base-line.

When such an asymmetrical curve is obtained it may be questioned whether we know that the boundary is really composite and that the asymmetry is not due to some obscure mechanical factor acting on a homogeneous boundary. The evidence against this is (1) that the base of the curve is much too broad for a homogeneous boundary of the same sedimentation constant; (2) that in several experiments with human serum we have found an asymmetrical $A+X$ curve in the early exposures which separated later into two symmetrical curves; (3) in experiments with certain concentrated globulin solutions the depression of the boundary diffusion is much greater than in the case of undiluted serum and no apparent asymmetry of the curve is present.

The only method we know of attempting to resolve graphically such a composite curve as that shown in Fig. 2c is to locate the points "a" and "c", delete the portion of curve between these points and try to reconstruct the ideal component curves by consideration of symmetry. The procedure is only possible when the portion of curve to the left of "a" is substantially more than half the whole A curve, and even then the accuracy must be very limited. In the case of undiluted cow serum we have usually found that it is possible to reconstruct the A curve in an apparently reproducible way, but the X curve cannot be satisfactorily drawn. We have, therefore, obtained a very approximate value for the area of the X component curve by subtracting the area of the symmetrical A curve from the total boundary area.

The general results of the first experiment are collected in Table I.

As in the previous paper we have obtained a comparative value for the discrepancy between measured and calculated protein concentration (entitled

Table I. *Results obtained with undiluted cow serum.*

	Average $(n_1 - n_0)$ calculated* $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w,20} \cdot 10^{13}$
A fraction	0·00952	4·76	2·67
X fraction	0·00253	1·27	3·2 ± 0·3
G fraction	0·00249	1·25	4·60
Total protein	0·01454	7·28	—

Total $(n_1 - n_0)$, by refractometric measurement on the serum and its dialysate, and correction of the result to $\lambda = 366 m\mu$ ... ... 0·0153

Total protein concentration, measured... ... ... 7·64 g./100 ml.

* These values are obtained from the area of the component curves on the sedimentation diagram by means of equation (3) of the previous paper [1935]. They represent the refraction increments of the individual fractions. The value for the total protein is the sum of the calculated individual values and represents, therefore, the calculated refractive equivalent of the total area of the sedimentation curve.

"'% diff.' total protein") by subtracting the value for the total protein refraction increment calculated from the area of the sedimentation diagram from that obtained by measurement on the serum and its dialysate, and expressing the difference as a percentage of the latter.

"% diff." in total protein values ... ... −5·2 %.

The individual values for the *A* and *X* fractions are only to be regarded as approximate for the reasons already discussed, but the combined value is exact to the limits of the experimental accuracy of the method. The calculated proportion of the total protein which is represented by the *G* fraction is 17·1 %. The *A* boundary sediments to the bottom of the cell with a steady, but very slight, diminution in the breadth of the boundary, thus giving rise to a negative spreading coefficient.[1] We regard this as evidence in favour of the dimensional homogeneity of the molecules of the *A* fraction. The *G* boundary spreading coefficient can only be determined with a very low degree of accuracy and since, in any case, the values are bound to vary with the concentration of *A* molecules, and, therefore, have little significance, we shall ignore them.

Exp. 2. Undiluted cow serum heated to 56° for 2½ hours. The object of this experiment was to find if any change in the molecular dimensions of the serum proteins accompanies the destruction of the complementary substance in immunological reactions.

A different 2·0 mm. cell from that used in Exp. 1 was employed in this case, and an optical scale distance of 1·38 cm. was found suitable. Mean centrifugal

Table II. *Results for heat-inactivated cow serum.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{w,20} \cdot 10^{13}$
A fraction	0·00968	4·85	2·68
X fraction	0·00198	0·99	3·2 ± 0·3
G fraction	0·00284	1·42	4·94
Total protein	0·01450	7·26	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$ ... ... 0·0153

Total protein concentration, measured ... ... 7·64 g./100 ml.

"% diff." in total protein values ... ... −5·5 %

Calculated proportion of *G* fraction ... ... 19·6 %

Spreading coefficient of *A* boundary ... ... Negative force = 255,000 times gravity. $T_f = 33{\cdot}5°$. $T_l = 35{\cdot}6°$. The photographic arrangements were the same as in Exp. 1. The curves are all so closely similar in shape to those in Exp. 1 that we have not reproduced a specimen here. The results are shown in Table II.

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[1] The spreading coefficient is defined in the previous paper and is calculated according to equation (8) of that paper.

The only change which might be a possible accompaniment of the heat-inactivation process is an increase in the concentration of the *G* fraction with a compensatory decrease in the concentration of the *X* fraction. The change, however, is not great enough to enable this conclusion to be definitely drawn. In all other respects the data for the normal and heat-inactivated sera are identical.

Exp. 3. The effect of simple dilution on the sedimentation diagram of cow serum. In the first experiment 1 volume of cow serum was diluted with 4 volumes of 1 % sodium chloride and the solution was examined in a 2·0 mm. cell. Mean centrifugal force = 235,000 times gravity. Optical scale distance = 10·14 cm. $T_f = 32{\cdot}2°$. $T_l = 33{\cdot}6°$. Photographic enlargement factor = 0·993. The diagram shown in Fig. 3 was obtained from an exposure taken 84 mins. after reaching full speed, and Table III gives the results.

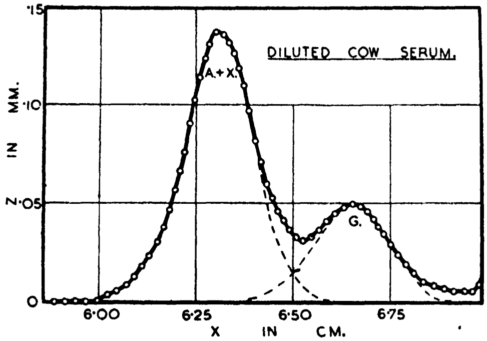

Fig. 3.

Table III. *Results obtained for cow serum diluted with 4 volumes of 1 °/o sodium chloride.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·0018	0·92	4·23
G fraction	0·0007	0·37	6·63
Total protein	0·0025	1·29	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$	0·00306
Total protein concentration, measured	1·53 g./100 ml.
"% diff." in total protein values	− 18·0 %
Calculated proportion of *G* fraction	28·0 %
Spreading coefficient of *A* boundary	$6{\cdot}1 \times 10^{-7}$

Owing to the increased spreading of the boundaries it is impossible to say whether the *A* fraction is homogeneous or consists of *A* + *X* fractions. It will be noted that there has been a substantial relative increase in the concentration of the *G* fraction and that the percentage discrepancy between measured and calculated $(n_1 - n_0)$ has increased considerably as compared with the previous two experiments.

In a second experiment the serum was examined after dilution with 7 volumes of 1 % sodium chloride. A 4·0 mm. cell was used. Optical scale distance = 8·14 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 31{\cdot}7°$. $T_l = 34{\cdot}5°$. Photographic enlargement factor = 0·996.

Table IV. *Showing the results obtained for cow serum diluted with 7 volumes of 1 °/₀ sodium chloride.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00117	0·59	4·43
G fraction	0·00043	0·22	6·70
Total protein	0·00160	0·81	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366\,m\mu$ ... ... 0·00201
Total protein concentration, measured ... ... 1·01 g./100 ml.
"% diff." in total protein values ... ... −20·4 %
Calculated proportion of *G* fraction ... ... 27·2 %
Spreading coefficient of *A* boundary ... ... $7{\cdot}7 \times 10^{-7}$

The relative concentration of the *G* fraction has not been increased by this further dilution and it appears probable, therefore, that the maximum "globulin" content of the serum obtainable by ultracentrifugal analysis is approximately 28 %. This will be referred to in a later paper where we shall have occasion to discuss the albumin and globulin concentrations in this serum obtained by precipitation methods. It is also of interest that the percentage discrepancy between measured and calculated $(n_1 - n_0)$ should again be so large in comparison with the case of undiluted serum. It suggests either that simple dilution of cow serum leads to the production of polydisperse protein or that in some way our technique is not exactly the same in the examination of concentrated and dilute solutions.

Exp. 4. Cow serum dialysed against 1 °/₀ sodium chloride. A sample of the cow serum was dialysed against several changes of 1 % sodium chloride until ionic equilibrium was obtained. During the process a slight dilution of the serum occurred. Cell thickness = 2·0 mm. Optical scale distance = 1·69 cm. Mean centrifugal force = 255,000 times gravity. $T_f = 32{\cdot}7°$. $T_l = 36{\cdot}9°$.

Table V. *Results obtained for cow serum dialysed against 1 °/₀ sodium chloride.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00852	4·26	3·09
X fraction	0·00135	0·67	—
G fraction	0·00225	1·13	5·07
Total protein	0·01212	6·06	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366\,m\mu$ ... ... 0·0133
Total protein concentration, measured ... ... 6·66 g./100 ml.
"% diff." in total protein values ... ... −8·9 %
Calculated proportion of *G* fraction ... ... 18·5 %
The spreading coefficient of the *A* boundary in this case is significantly zero.

We may note here the slightly increased sedimentation constants and boundary spreading coefficients as compared with the untreated serum in Exp. 1. These increases may be due to the dilution which occurred inadvertently during the dialysis.

It is also possible that the rather low value of $(n_1 - n_0)$ for the X fraction is due to the slightly increased spreading at the A and X boundaries which makes the graphical separation of the two curves more uncertain than usual. In general, however, we conclude from this experiment that dialysis against 1 % sodium chloride does not affect significantly the protein sedimentation diagram of undiluted cow serum.

Exp. 5. Cow serum dialysed against 0·1 °/₀ sodium chloride. The dialysed serum was examined in a 2·0 mm. cell with an optical scale distance of 2·28 cm. The fact that we were able to use this greater scale distance indicated early in the experiment that a significant change had occurred in the sedimentation diagram. Mean centrifugal force = 255,000 times gravity. $T_f = 32{\cdot}9°$. $T_l = 25{\cdot}2°$.

Table VI. *Results for cow serum dialysed against 0·1 °/₀ sodium chloride.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00786	3·93	2·76
X fraction	0·00167	0·84	—
G fraction	0·00304	1·52	5·45
Total protein	0·01257	6·29	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366\,m\mu$ ... ... 0·0134
Total protein concentration, measured ... ... 6·70 g./100 ml.
"% diff." in total protein values ... ... −6·0 %
Calculated proportion of G fraction ... ... 24·2 %
Spreading coefficient of A boundary ... ... $0{\cdot}6 \times 10^{-7}$

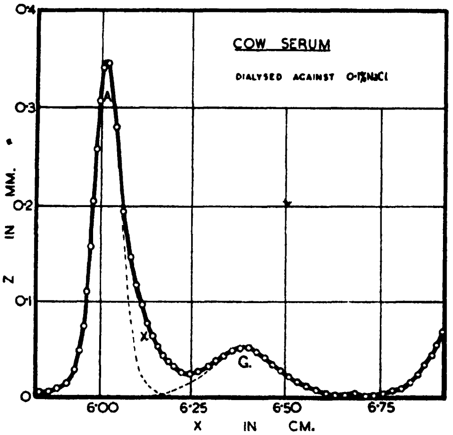

Fig. 4.

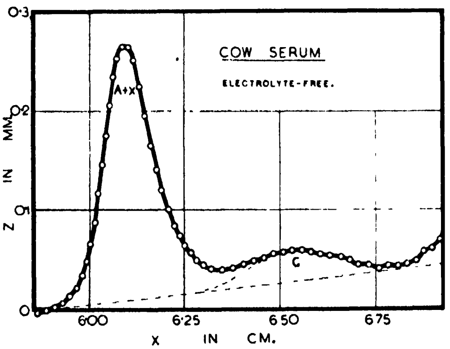

Fig. 5.

We reproduce in Fig. 4 the curve of an exposure taken 56½ mins. after reaching full speed. It will be seen on comparison with the curve for untreated serum in Fig. 1 that the two curves have the same general shape, but as the calculations show, the G curve in Exp. 4 has a definitely greater area than the G curve in Exp. 1, and the A curve in Exp. 4 has a smaller area than the A curve in Exp. 1. The sedimentation velocity of the G fraction appears to be abnormally high. The increase in the boundary spreading coefficient of the A fraction was expected since charged proteins tend to diffuse more rapidly in the presence of lower concentrations of diffusible electrolytes. In order to find if further reduction in the concentration of diffusible electrolytes would give rise to a further increase in the G fraction we examined next electrolyte-free cow serum.

Exp. 6. Electrolyte-free cow serum. The cow serum was dialysed for several days in a collodion bag against distilled water during which procedure a rather scanty flocculum of euglobulin collected at the bottom of the bag. The supernatant solution was then centrifuged in a 2·0 mm. cell. Mean centrifugal force = 255,000 times gravity. Optical scale distance = 3·78 cm. $T_f = 33{\cdot}8°$. $T_l = 35{\cdot}8°$.

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$ ...	0·011
Total protein concentration, measured	5·51 g./100 ml.

Fig. 5 shows the curve of an exposure taken 52½ mins. after reaching full speed. The base-line is sloping probably because of the high potential gradient set up in the solution by the movement of charged protein in an electrolyte-free medium. It is, therefore, not possible to make calculations of $(n_1 - n_0)$ from this curve which will have any precise significance, but we may obtain approximate values for the sedimentation constants of the A and G fractions. The values are:

$S_{W,20}$ for the A fraction	$3{\cdot}4 . 10^{-13}$
,, G ,, ...	$7{\cdot}4 . 10^{-13}$

It is impossible to say whether any X fraction is present or not.

The high value of $S_{W,20}$ for the G fraction conforms with the high value of the previous experiment. It may be due to charge effects or to an increase in the size of the G molecules. We may also notice in Fig. 5 the marked increase in the degree of spreading at both boundaries. In connection with certain arguments discussed in the previous paper concerning the charge on the proteins in untreated serum, this phenomenon certainly suggests that both fractions in electrolyte-free serum are charged. It may not be inferred, however, that the same is true of untreated serum until we have more evidence that the constitution of the soluble protein in serum is not affected by the removal of electrolytes.

Exp. 7. Cow serum dialysed against M/*20 acetate buffer.* p_H *4·6.* Cell thickness = 2·0 mm. Mean centrifugal force = 250,000 times gravity. Optical scale distance = 1·24 cm. $T_f = 33{\cdot}4°$. $T_l = 35{\cdot}9°$. The results are shown in Table VII.

Table VII. *Results with cow serum dialysed against* M/*20 acetate buffer.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} . 10^{13}$
A fraction X fraction	0·00983	4·92	3·29
G fraction	0·00236	1·18	5·10
Total protein	0·01219	6·10	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$	0·0125
Total protein concentration, measured	6·26 g./100 ml.
"% diff." in total protein values	−2·4 %
Calculated proportion of G fraction	19·4 %
Spreading coefficient of A boundary	$0{\cdot}3 \times 10^{-7}$

Owing to a certain amount of leakage from the cell at the beginning of this run the sedimentation path available was shorter than usual, with the result that the presumably merged A and X boundaries reached the bottom of the cell before any detectable separation of the two had time to occur. It was therefore only possible to calculate combined values for $(n_1 - n_0)$ and $S_{W,20}$, for this boundary. In all other respects, and taking into account the slight dilution during dialysis, we regard the curve as showing no significant points of difference from the curve of untreated cow serum.

Exp. 8. Cow serum dried and redissolved. A portion of the serum was rapidly dried under reduced pressure in a desiccator containing phosphorus pentoxide and then mixed with the same volume of water as there was serum originally. In spite of stirring the mixture gently at intervals for 24 hours a rather bulky greyish-brown residue remained. The clear solution after centrifuging off the insoluble material was then dialysed under pressure against 1 % sodium chloride until equilibrium was reached and the total protein concentration was approximately the same as that of the original serum. The solution was examined in a 2·0 mm. cell. Mean centrifugal force = 240,000 times gravity. Optical scale distance = 1·54 cm. $T_f = 33{\cdot}3°$. $T_l = 34{\cdot}4°$.

Fig. 6 shows the curve of an exposure taken 66 mins. after reaching full speed, and Table VIII shows the general results of the run.

Table VIII. *Results for cow serum which had been dried and redissolved.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{\mathrm{w},20} \cdot 10^{13}$
A fraction	0·00940	4·73	2·37
X fraction	0·00183	0·92	—
G fraction	0·00235	1·18	4·37
Total protein	0·01358	6·83	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366\,m\mu$	0·0161
Total protein concentration, measured	8·06 g./100 ml.
"% diff." in total protein values	−15·5 %
Calculated proportion of *G* fraction	17·3 %

The spreading coefficient of the *A* boundary has a negative value.

It will be seen from Table VIII and from Fig. 6 that the results generally are in rather close agreement with those for untreated cow serum, except that there is a greater disrepancy in the total protein values. The values of the sedimentation constant and spreading coefficient are in keeping with the slightly higher total protein concentration in this experiment than in Exp. 1, but the calculated value for $(n_1 - n_0)$ is definitely less. It appears, therefore, that, compared with the untreated serum, some 8–10 % more of the total protein in the redissolved serum is completely heterodisperse in the centrifugal field and does not show on the apparent base-line. It is of interest that, if we disregard this polydisperse protein and the insoluble protein left on redissolving the dried serum, the sedimentation characteristics of the remaining soluble protein after concentration and the concentration ratio of its constituent fractions should be so closely similar to those of the untreated parent serum.

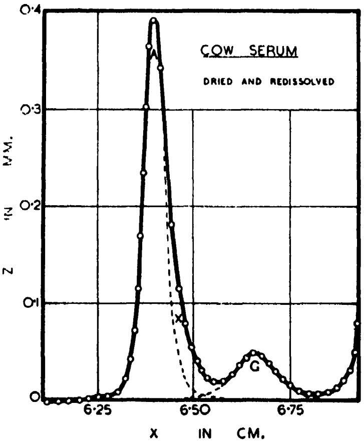

Fig. 6.

Exp. 9. Cow serum, dried, ether-extracted and redissolved. In order to investigate whether the serum lipoids have any influence on the character of the sedimentation diagram we have subjected a portion of the serum powder (prepared as in Exp. 8) to the action of warm ether in a Soxhlet extractor for 3 days. The residue in the extraction thimble was exposed to the laboratory atmosphere for a day to get rid of all traces of ether and then redissolved and dialysed against 1 % sodium chloride. As in the previous experiment there was a bulky residue of insoluble protein. The protein solution after dialysis was examined in a 2·0 mm. cell, using a mean centrifugal force of 255,000 times gravity. Optical scale distance = 2·88 cm. $T_f = 33·7°$. $T_l = 35·7°$.

Table IX. *Results for cow serum which had been dried, ether-extracted and redissolved.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00592	2·96	3·38
X fraction	0·00117	0·59	—
G fraction	0·00185	0·93	5·40
Total protein	0·00894	4·48	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$	0·0119
Total protein concentration, measured	5·96 g./100 ml.
"% diff." in total protein values	−24·8 %
Calculated proportion of *G* fraction	20·6 %
Spreading coefficient of *A* boundary	$1·0 \times 10^{-7}$

The most notable feature is the fact that almost one-quarter of the total protein present has failed to appear on the diagram. A discrepancy of the same order was found in Exp. 3 using diluted cow serum and there we could not exclude the possibility that an error might arise owing to the different conditions (cell thickness, scale distance, time of experiment *etc.*) under which a dilute and a concentrated solution are examined. In this case we feel justified in drawing the definite conclusion that a considerable amount of polydisperse protein is present because the conditions of the experiment are practically identical with those in Exps. 1, 2, 4, 5, and 7, in which an average loss of only 5·5 % is recorded. It is also interesting to find in this experiment that the refraction equivalent of the total area of the curve of the first exposure, *viz.* 0·0107, is much higher than in the later exposures where it has a constant value of 0·00894. The spreading coefficient of the *A* boundary also appears to be high, but this may be due to the rather low concentration of the homogeneous protein which is present.

It is again interesting to find that in spite of the protein which was lost in the form of the insoluble residue, and of the polydisperse protein which fails to show its presence on the sedimentation diagram, the remaining protein appears to be normal from the sedimentation point of view. We should have expected that the protein loss would fall more heavily on one or other of the fractions. We must conclude from this that the denaturation process affects each fraction in proportion to the concentration in which it is present, or else that the three fractions are in equilibrium and removal of part of one fraction is followed by an internal molecular rearrangement which restores the fractions to their normal relative concentrations. This latter possibility does not seem to us unlikely in view of the equilibrium which exists in mixtures of serum albumin and globulin. We also conclude from this experiment that the serum lipoids which are extractable with ether do not exert an essential influence on the character of the sedimentation diagram.

Exp. 10. Cow serum in the presence of ammonium sulphate. In a preliminary test on a sample of cow serum it was found that globulin precipitation began when 3·9 ml. saturated ammonium sulphate had been added to 10 ml. of serum. We then added to a 1 ml. sample of the serum 0·3 ml. saturated ammonium sulphate and examined the clear solution in the centrifuge. The object of the experiment was to find whether any marked change is induced in the sedimentation diagram by ammonium sulphate in amount insufficient to cause precipitation. Cell thickness = 2·0 mm. Optical scale distance = 3·78 cm. Mean centrifugal force = 255,000 times gravity. $T_f = 33{\cdot}3°$. $T_l = 35{\cdot}4°$.

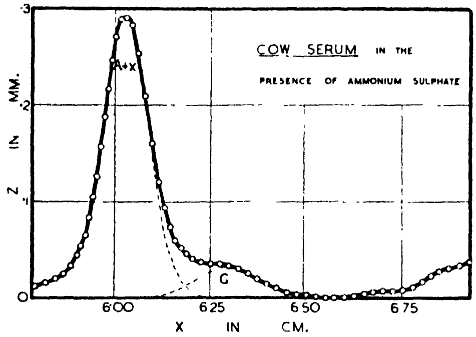

Fig. 7.

The curve shown (Fig. 7) was obtained from a photograph taken 88½ mins. after reaching full speed. This and other exposures show base-line irregularities which are due at least in part to sedimentation of the ammonium sulphate ions. Two fractions are present, and owing to the density and viscosity effects of the ammonium sulphate these sediment very slowly, the complete run requiring 4 hours. More blurring of the boundaries thus has time to occur with the result that no signs of a separation of the lighter fraction into *A* and *X* components can be seen. Also, owing to the abnormal conditions of sedimentation, we cannot say from the spreading coefficient of this boundary whether it is homogeneous or not.

Table X. *Results for cow serum in the presence of ammonium sulphate.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00618	3·09	3·07
G fraction	0·00053	0·27	5·36
Total protein	0·00671	3·36	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$	0·00109
Total protein concentration, measured	5·46 g./100 ml.
"% diff." in total protein values	−38·5 %
Calculated proportion of *G* fraction	7·9 %

The individual values of $(n_1 - n_0)$ for the *A* fraction are interesting, *viz.* 6·64 after 88½ mins., 5·64 after 155 mins. and 5·77 after 204 mins. The spreading coefficients are also uneven—*e.g.* calculated between the exposures at 88½ and 155 mins. = $2{\cdot}46 \,.\, 10^{-7}$, and between the exposures at 155 and 204 mins. = $0{\cdot}86 \,.\, 10^{-7}$. As already pointed out, owing to the slow sedimentation we cannot compare these values with the spreading coefficients which we have determined in other experiments. It is, however, interesting to find a large degree of boundary spreading with falling $(n_1 - n_0)$ in the upper part of the cell and a much smaller degree of spreading with constant $(n_1 - n_0)$ in the lower part of the cell. The whole picture, including the marked discrepancy between measured and calculated total $(n_1 - n_0)$ values, leaves little doubt that a large part of the protein in the solution is polydisperse. Mutzenbecher [1933] has also found that ammonium sulphate causes a dissociation of the serum protein molecules prior to precipitation.

It is also to be noted that the percentage of the total protein refraction increment due to the *G* fraction is very low. It would appear in this case (as distinct from the state of affairs when serum is dried and redissolved) that the loss of protein has fallen more heavily on the *G* fraction. This we would expect since it is only necessary to increase the concentration of ammonium sulphate to the point of precipitation and the globulin fraction practically disappears altogether from the diagram.

Exp. 11. Cow serum treated with ammonium sulphate. In this experiment a collodion dialysis bag filled with cow serum and closed was immersed in a large volume of saturated ammonium sulphate. In a few minutes the serum protein precipitated completely and was left so for 24 hours. The bag was then removed from the ammonium sulphate solution and placed in 1 % sodium chloride solution which was changed frequently until the dialysate was free from sulphate ions. It was verified that no protein leakage had occurred through the bag, the change in the total protein refraction increment of the serum—from 0·00141 to 0·00132—being due to incidental dilution during the dialysis process. The serum was then examined in the same cell as was used in the previous experiment. Mean centrifugal force = 255,000 times gravity. Optical scale distance = 2·28 cm. $T_f = 32{\cdot}3°$. $T_l = 33{\cdot}7°$.

Table XI. *Results for cow serum treated with ammonium sulphate.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} . 10^{13}$
A + *X* fraction	0·00975	4·88	3·11
G fraction	0·00174	0·87	4·71
Total protein	0·01149	5·75	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$ ... ... 0·0143
Total protein concentration, measured ... ... 7·13 g./100 ml.
"% diff." in total protein values ... ... −20 %
Calculated proportion of *G* fraction ... ... 15 %

The curve shown in Fig. 8 was obtained from a photograph taken 116½ mins. after reaching full speed. At the apex of the *A* curve there is definite evidence that this fraction is not homogeneous but consists of two molecular species present in approximately equal concentrations. The later exposures from this experiment show the same signs of an early process of resolution of the *A* curve into two curves of approximately equal areas—a process which is, however, still incomplete when the boundary reaches the bottom of the cell. The spreading coefficient of this boundary is $0{\cdot}25 . 10^{-7}$, which is in keeping with a heterogeneous fraction in view of the negative values obtained with the untreated serum.

In Fig. 9 we have shown the curve of a late exposure in Exp. 1 (untreated cow serum), and we have also superimposed on it the curve of the corresponding exposure from this experiment. Owing to the steeper boundary gradient in

Exp. 1 the two experiments were carried out with different scale distances. In order to make the two curves strictly comparable, therefore, we have recalculated each scale line position for the exposure in Exp. 11 to the same scale distance as used in Exp. 1, and these recalculated values are plotted in Fig. 9.

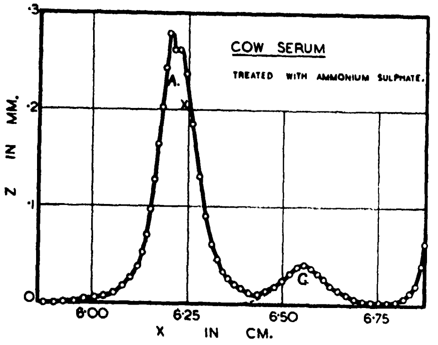

Fig. 8.

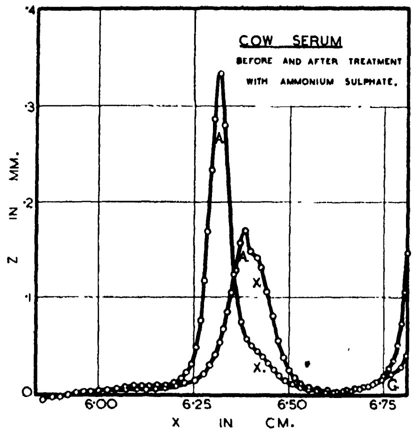

Fig. 9.

There still remains a difference in the total areas of the two curves owing to the slightly lower total protein area in Exp. 11. The difference is too small, however, to affect significantly the shape of the curves. A comparison of the two demonstrates the difference in the relative concentrations of the *A* and *X* molecules in the two cases much more strikingly than the figures which we have been able to give for the spreading coefficient of the *A* boundary in the two experiments.

We have generally preferred to base our conclusions with regard to the homogeneity of a sedimenting fraction on measurements of the actual boundary spreading which occurs between two levels in the cell rather than on measurements of the dimensions of one boundary curve after a given time. We regard this as more reliable because the dimensions of any particular boundary curve are dependent, among other things, on the time required to accelerate the centrifuge to full speed, and, possibly, also on the degree of convectional disturbances at the meniscus during this period. Also, of course, the dimensions are directly dependent on the scale distance, thickness of cell and photographic enlargement factor, which differ between experiments. On the other hand the effect of these latter variables may be eliminated by recalculating the scale line positions for one experiment to the conditions of the other as we have done here. In all our experiments also we have employed, as far as possible, a standard acceleration period for the centrifuge and approximately the same maximum speeds, and it has been our experience that boundary curves with significantly aberrant dimensions do not occur.

With due consideration of the many difficulties in the way of an exact comparison of the dimensions of curves from different experiments, we feel justified in including the evidence in Fig. 9 of gross changes in the dimensions of the curve together with that of the shape of the curve at the apex and the value of the boundary spreading coefficient as support for the view that ammonium sulphate causes an irreversible change in cow serum. The nature of the change may be defined as such that the concentration of the *X* molecular fraction is increased to a value comparable with that of the A fraction, and that the increase is at the expense of the A fraction. It will be shown in the next section that the same appears to be true for horse serum.

SECTION 2. THE SEDIMENTATION DIAGRAM OF NORMAL HORSE SERUM.

In the previous paper [McFarlane, 1935] we have published certain data and a curve from an experiment with fresh untreated horse serum. We pointed out there that the curve was not symmetrical, but to avoid confusing the special issue of that work, which it did not affect in essential principle, we passed over the question of this asymmetry and discussed the serum as a two-component mixture. In the light of the experiments which will now be reported it is necessary to regard horse serum with cow serum as having a heterogeneous lighter molecular fraction consisting of two component fractions which are only imperfectly separable in the centrifugal field. In this section we shall report the results of some experiments on horse serum which were undertaken largely in order to enable a comparison of the results with those obtained for cow serum.

Exp. 12. Untreated horse serum. We are indebted to Prof. Robin Fåhraeus for the supply of this serum from a horse which had not been immunised and had no signs or history of disease. The results of this experiment are taken from the previous paper and given here for purposes of comparison with later experiments. On Fig. 3 (*a*) of the paper referred to there will be found the curve of an exposure taken 117 mins. after reaching full speed. The experimental conditions were: Cell thickness = 2·0 mm. Optical scale distance = 2·14 cm. Mean centrifugal force = 230,000 times gravity. $T_f = 32{\cdot}3°$. $T_l = 34{\cdot}0°$.

Table XII. *Results for untreated horse serum.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00854	4·32	2·94
X fraction	0·00206	1·04	—
G fraction	0·00269	1·34	4·35
Total protein	0·01329	6·70	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366\,m\mu$	0·0144
Total protein concentration, measured	7·25 g./100 ml.
"% diff." in total protein values	−8·9 %
Calculated proportion of G fraction	20·2 %
Spreading coefficient of A boundary	$0{\cdot}8 \times 10^{-7}$

Exp. 13. Undiluted horse plasma. A sample of blood from the same horse and taken at the same time as the serum in Exp. 12 was collected in a vessel containing sufficient dry sodium oxalate to make an approximately 0·5 % solution in the plasma. The blood corpuscles were centrifuged off 2 hours later and the clear plasma examined in the same 2·0 mm. cell as was used in the previous experiment. Optical scale distance = 2·14 cm. Mean centrifugal force = 240,000 times gravity. $T_f = 32{\cdot}9°$. $T_l = 34{\cdot}1°$.

Fig. 10 shows the curve of an exposure taken 75 mins. after reaching full speed, and the results are shown in Table XIII.

A comparison of the results in Exps. 12 and 13 and of the curve of Fig. 10 with that of Fig. 3 (*a*) of the previous paper reveals a very close similarity in the characteristics of the serum and plasma of the same animal. The additional protein in the diagram of the plasma, corresponding to the fibrinogen which is removed in the clot, is represented apparently by slight increases in all

Table XIII. *Results for undiluted horse plasma.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00868	4·39	2·86
X fraction	0·00240	1·21	—
G fraction	0·00345	1·72	4·44
Total protein	0·01453	7·32	—

Since it was considered inadvisable to dialyse the plasma no measured value for $(n_1 - n_0)$ was obtained.
Calculated proportion of *G* fraction ... ... 24 %
Spreading coefficient of *A* boundary ... ... $0 \cdot 8 \times 10^{-7}$

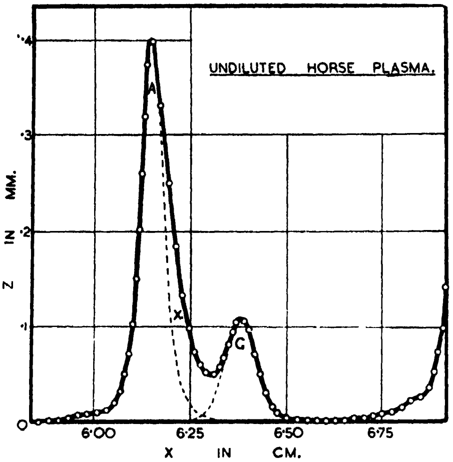

Fig. 10.

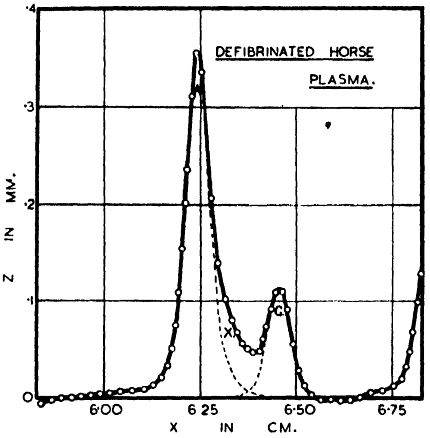

Fig. 11.

the fractions, but the most definite increase is clearly associated with the *G* molecule. We know from previous work that if some globulin were added to the serum used in Exp. 12 it would cause an increase both in the *G* curve and in the composite $A + X$ curve owing to the equilibrium which exists between those fractions. It appears possible then that the fibrinogen of oxalate plasma is present with a molecular weight approximately the same as that of the globulin fraction and that it takes part in the general protein equilibrium reaction of the serum. This would explain the slight increases in the *A* and *X* fractions. Without some such explanation as this we must assume that fibrinogen is not a single substance, but consists of two or three fractions of the same sedimentation constants as the corresponding serum fractions. As a result of this experiment we can say definitely that no new fibrinogen molecule appears in the diagram of the plasma. It is interesting to note in this connection that Verney [1926] found no significant difference in the osmotic pressure per g. of total protein in the case of serum and plasma from the same human subject, indicating that fibrinogen has approximately the same mean molecular weight as the mean molecular weight of the serum proteins.

Exp. 14. Defibrinated horse plasma. We have defibrinated a sample of oxalate plasma from another horse by a usual procedure, *viz.* by adding slightly more solid calcium chloride than is required to precipitate all the oxalate and then stirring vigorously with a glass rod. The shreds of fibrin were centrifuged off and the clear defibrinated plasma examined in the centrifuge in a 2·0 mm. cell. Optical scale distance = 1·54 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 33{\cdot}4°$. $T_l = 34{\cdot}5°$.

Table XIV. *Results for defibrinated horse plasma.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·01100	5·56	2·68
X fraction	0·00231	1·17	—
G fraction	0·00349	1·74	3·66
Total protein	0·01680	8·47	—

The preparation again was not dialysed before the experiment so that no measurements of total protein refractivity were made.

Calculated proportion of *G* fraction	20·8 %
Spreading coefficient of *A* boundary	$0{\cdot}3 \times 10^{-7}$

The curve shown in Fig. 11 is from an exposure taken 120½ mins. after reaching full speed.

It is interesting to find that, in spite of the fact that this serum contains considerably more protein than that of Exp. 12, the sedimentation diagram in the two cases is closely similar. The diminished sedimentation constants and spreading coefficient of the *A* boundary in the latter experiment are in keeping with the greater protein concentration.

Exp. 15. Horse serum treated with ammonium sulphate. We have undertaken this experiment in view of the change which had been noticed in the sedimentation diagram of cow serum as a result of treatment with ammonium sulphate. The experimental details were exactly as in Exp. 11 with cow serum. A specimen of the horse serum used in Exp. 12 was dialysed against a saturated solution of ammonium sulphate for 24 hours in a closed collodion bag. Thereafter, the serum was dialysed against changes of 1 % sodium chloride until all sulphate ions had disappeared from the dialysate. Cell thickness = 2·0 mm. Mean centrifugal force = 250,000 times gravity. $T_f = 33{\cdot}6°$. $T_l = 36{\cdot}5°$.

Table XV. *Results for horse serum treated with ammonium sulphate.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366\,m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00836	4·23	3·43
G fraction	0·00290	1·44	5·01
Total protein	0·01126	5·67	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366\,m\mu$	0·00130
Total protein concentration, measured	6·54 g./100 ml.
"% diff." in total protein values	−13 %
Calculated proportion of *G* fraction	25 %
Spreading coefficient of *A* boundary	$2{\cdot}2 \times 10^{-7}$

The curve shown in Fig. 12 was obtained from a scale photograph taken 95½ mins. after reaching full speed. It will be seen that this *A* curve shows no signs of dividing into two as was the case with cow serum. It will have been noted, however, in the horse serum experiments that we have never found any negative values for the spreading coefficient of the *A* boundary such as occurred frequently in the cow serum experiments. In general, the degree of *A* boundary spreading is greater in the horse serum experiments and this may be due to the lower total protein concentration in the horse serum and the slightly greater proportion of *G* fraction. We believe this increased boundary spreading to be the reason for the apparent monodispersity of the *A* boundary in this experiment.

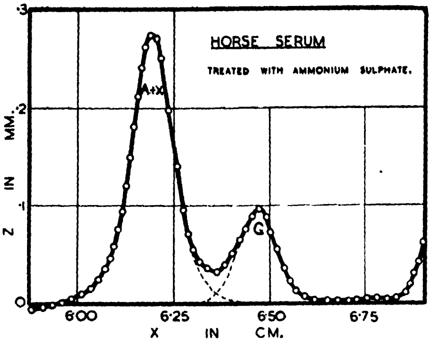

Fig. 12.

On the other hand, the *A* curve in all exposures is perfectly symmetrical, the boundary spreading coefficient appears to be abnormally high, and if the curve in Fig. 12 is replotted to the same scale distance as the curve for the original untreated serum it is seen that there has been a marked change in the general dimensions of the boundary curve similar to the change shown for cow serum in Fig. 9.

In the previous paper we have described an experiment in which albumin and globulin were prepared from this serum by fractionation with ammonium sulphate and then mixed together to form a so-called "synthetic" horse serum. A curve from this experiment is shown in Fig. 3 (*b*) of that paper, and in it, as in all the curves of that experiment, the *A* boundary curve is perfectly symmetrical.

For these reasons we believe that in the treatment of horse serum as well as cow serum with strong solutions of ammonium sulphate a definite change, which the ultracentrifuge is just able to detect and little more, occurs in the composition of the lighter molecular fraction.

We may not attach any particular significance to the high proportion of *G* fraction in this experiment because it will be noted that slight dilution of the serum has occurred during the dialysis. The data of the previous paper, supported by further evidence derived from the next experiment, show that the concentration relationship of the fractions in horse serum is exceedingly sensitive to changes in the total protein concentration—much more so than in the case of cow serum.

Exp. 16. Horse serum diluted with 3 volumes of 1 °/₀ sodium chloride. A sample of the serum used in Exp. 12 was diluted with 3 volumes of a 1 % solution of sodium chloride and examined in a 2·0 mm. cell using an optical scale distance of 8·78 cm. Mean centrifugal force = 250,000 times gravity. $T_f = 32{\cdot}2°$. $T_l = 33{\cdot}2°$. Photographic enlargement factor = 0·996.

The curve in Fig. 13 was obtained from an exposure taken 48 mins. after reaching full speed. In the resolution of this curve into *A* and *G* components

Table XVI. *Results with horse serum diluted with 3 volumes of 1 °/₀ sodium chloride.*

	Average $(n_1 - n_0)$ calculated $\lambda = 366 m\mu$	Calculated protein concentration g./100 ml.	Average $S_{W,20} \cdot 10^{13}$
A fraction	0·00171	0·87	4·22
X fraction	0·00013	0·07	—
G fraction	0·00105	0·52	6·51
Total protein	0·00289	1·46	—

Total $(n_1 - n_0)$, measured and corrected to $\lambda = 366 m\mu$ ... ... 0·00353
Total protein concentration, measured ... ... 1·78 g./100 ml.
"% diff." in total protein values ... ... 18 %
Calculated proportion of *G* fraction ... ... 36 %
Spreading coefficient of *A* boundary ... ... $6·6 \times 10^{-7}$

it appears that a small part of the total area cannot be included in the area of the symmetrical component curves. This small area lying in the region between the two curves we have called the *X* fraction in Table XVI, but if, like the *X* fraction in the undiluted serum, it has an average sedimentation constant very close to that of the *A* fraction it is probable that what we have classed as "*A*" fraction is not homogeneous but contains significant quantities of an *X* fraction. With the markedly increased boundary spreading in dilute protein solutions it is not possible to attempt a resolution of the lighter component into *A* and *X* fractions with any degree of accuracy. The value which we give in Table XVI for the *X* fraction is, therefore, merely an estimate of the refractive equivalent of the asymmetrical area not included in the *A* and *G* curves and has a doubtful connection with the *X* fraction of undiluted serum.

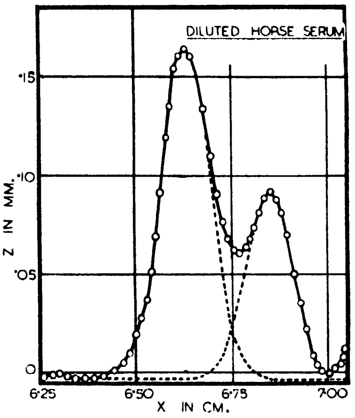

Fig. 13.

A comparison of the results of this experiment with those of Exp. 3 will show that the increase in the proportion of the *G* fraction on diluting cow serum is much less than the increase on diluting horse serum to the same extent. No doubt this is related to the higher albumin : globulin ratio in cow serum obtained by precipitation methods.

SECTION 3. THE SEDIMENTATION DIAGRAM OF NORMAL HUMAN SERUM.

Our first experiment using fresh undiluted human serum in a 2·0 mm. cell gave rise to a curve of the same general three-component type as the curves of undiluted cow and horse serum, but with two points of difference. The concentration of the *G* fraction was considerably smaller than in those sera, and the composite *A*+*X* curve showed marked distortion of the type illustrated in Fig. 2D. In attempts to reduce this distortion we decided to try diluting the serum with small amounts of 1 % sodium chloride such as would not be expected to give rise to any significant change in the relative concentrations of the fractions.

Exp. 17. ***Normal human serum diluted with one-third its volume of 1 °/₀ sodium chloride.*** A 2·0 mm. cell was used. Optical scale distance = 1·54 cm. Mean centrifugal force = 240,000 times gravity. $T_f = 33{\cdot}2°$. $T_l = 35{\cdot}1°$. The results are shown opposite the reference initials J.S.P. in Table XVII and the curve in Fig. 14 is from a photograph taken 92 mins. after reaching full speed. It will be seen that the concentration of the *G* fraction is small, and that a certain amount of distortion is still present in the $A + X$ curve. In the exposure taken 150 mins. after reaching full speed, however, the *A* and *X* curves had become sufficiently separated for each boundary to be free of the distorting influence of the other and we find two symmetrical curves. We are able, therefore, to estimate with considerable accuracy the sedimentation constant and concentration of the *X* fraction in this serum.

Exp. 18. The same normal human serum (J.S.P.) diluted with an equal volume of 1 °/₀ sodium chloride. The same cell was used as in Exp. 17. Optical scale distance = 2·74 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 30{\cdot}3°$. $T_l = 32{\cdot}4°$. The results are also shown in Table XVII.

The curve in Fig. 15 was obtained from a photograph taken 83 mins. after reaching full speed, and shows that practically no change has occurred in the concentration of the *G* fraction, but owing to the increased boundary spreading it is now impossible to obtain an accurate graphical separation of the *A* and *X* curves.

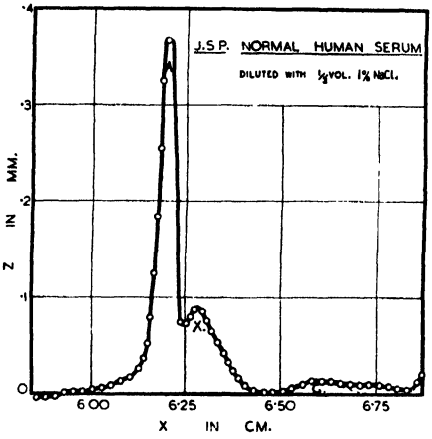

Fig. 14.

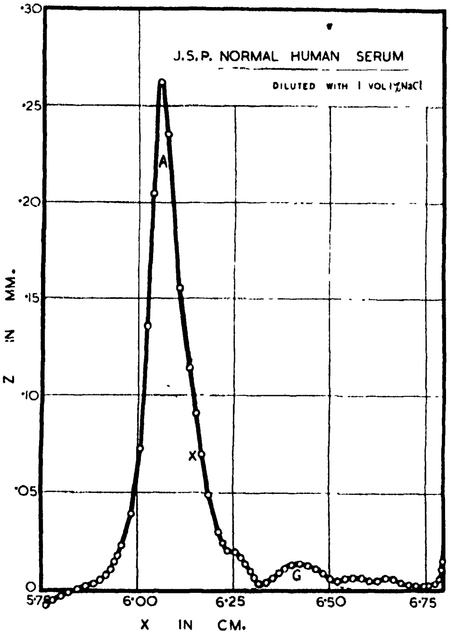

Fig. 15.

Since small additions of 1 % sodium chloride do not appear to affect significantly the concentrations of the fractions in human serum it appears to be permissible to adopt the method of slight dilution in order to obtain a more satisfactory separation of the *A* and *X* curves. It is probable that the addition to a normal serum of more than half its own volume of 1 % sodium chloride will defeat the purpose for which it is added and give rise to a curve of the type shown in Fig. 15 which cannot be used to obtain accurate concentration values for the *A* and *X* fractions. Somewhere between this dilution and a state of no dilution there must be an optimum total protein concentration for symmetrical separation of the *A* and *X* curves. It does not appear that we can predict this dilution beforehand in any particular case because in addition to the total protein concentration, which we can control, there is the concentration ratio of the *A* and *X* fractions, which we cannot alter. We believe from our experiences to date that under the present conditions of an ultracentrifuge experiment the concentration ratio of the *A* and *X* fractions may influence the degree of distortion of the curves. We suspect, indeed, that it is the higher average proportion of *X* fraction in normal human serum which gives rise to the more pronounced distortion in this curve than in the curves of normal cow or horse serum. Our evidence for this, however, is mainly presumptive at present and it is possible that the true reason lies in slight differences in the sedimentation constants of the *A* and *X* proteins in the serum of different species—differences which are too small to be measurable by present methods. It is also possible that even slight changes in the total protein concentration of a serum may cause small changes in the concentration ratio of the *A* and *X* fractions, which, although not great enough to be measurable in the diagrams so far obtained, may nevertheless influence the degree of distortion of the curve.

Altogether, the variable factors are so numerous and the data available at present are so scanty that we have no choice but to resort to a method of trial and error in order to find the best dilution at which to examine a human serum. A factor which we have not mentioned, and which from several points of view must play an important part in the separation of the *A* and *X* curves, is the centrifugal force used. Here again, however, it is not possible to be sure of the nature of the effect, but it seems probable that with higher centrifugal forces the degree of boundary spreading will be reduced and the rate of separation of the two boundaries increased so that we may hope to obtain a satisfactory separation of the *A* and *X* fractions even using undiluted serum. Fortunately, also, much higher centrifugal forces have recently been obtained and a new rotor for serum work is under construction, which Prof. Svedberg hopes will enable us to take advantage of these forces and even permit at the same time of a slight increase in the height of fluid column in the cell. When this rotor is available we hope that it may be possible to investigate as a separate problem the effect of a wide range of centrifugal forces on the diagram of a normal human serum. In the following experiments we have had to employ a standardised centrifugal force in order to make the results more strictly comparable. We have, however, employed various dilutions of human serum so that the curves in some cases are rather more symmetrical than in others.

Exp. 19. The same normal human serum (J.S.P.) diluted with 7 volumes of 1 °/₀ sodium chloride. A 4·0 mm. cell was used and an optical scale distance of 8·0 cm. Mean centrifugal force = 235,000 times gravity. $T_f = 30{\cdot}3°$. $T_l = 32{\cdot}4°$. Fig. 16 shows the curve of a scale photograph taken 51 mins. after reaching full speed, and the results are recorded in Table XVII. The concentration of the *G* fraction is still remarkably small, especially in comparison with diluted cow and horse serum. We have in another experiment examined the same serum diluted with 31 volumes of 1 % sodium chloride. The curves obtained all show irregularities which may be due to a disintegration of the protein molecules, or, on the other hand, may arise from convection effects in the cell. We do not, therefore, publish the results but we may state, from the general appearance of the curve, that even at this dilution no dramatic appearance of globulin molecules takes place.

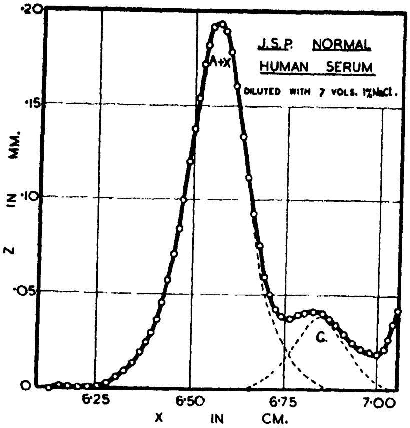

Fig. 16.

Table XVII. *Results obtained in the investigation of a range of normal human sera.*

Serum	Dilution in vols. serum: vols. 1 % NaCl	Cell thickness mm.	Optical scale dist. cm.	Average $S_{W,20} . 10^{13}$ A mol.	G mol.	X mol.
J.S.P.	3 : 1	2·00	1·54	2·63	4·47	3·14
J.S.P.	1 : 1	2·00	2·74	3·30	5·15	—
J.S.P.	1 : 7	4·00	7·64	4·48	6·64	—
W.L.	5 : 2	2·00	1·98	2·90	5·14	3·40
C.A.	5 : 2	2·00	1·38	2·69	5·24	3·20
O.D.B.	3 : 1	2·00	1·24	2·60	5·05	3·02
R.N.	5 : 2	2·00	1·69	3·01	5·33	3·31
A.G.P.	5 : 2	2·00	1·98	3·03	4·86	3·20
M.S.	3 : 1	2·00	1·84	3·30	5·95	—
I.S.	Undil.	2·00	2·14	2·88	5·39	—
A.McF.	1 : 1·25	2·00	3·64	3·92	5·67	—
O.Q.	1 : 1·25	2·00	3·64	3·54	5·47	—

Serum	Average calc. $(n_1 - n_0) . 10^3$ $\lambda = 366 m\mu$ A mol.	G mol.	X mol.	Total	Conc. G mol. % total protein	Calc. total $(n_1 - n_0) . 10^3$ for undil. serum	Total protein conc.* of undil. serum g./100 ml.	Spreading coeff. of A boundary cm.²/sec. 10^7
J.S.P.	8·97	1·10	2·70	12·77	8·6	17·03	8·43	0·4
J.S.P.	5·47	0·625	2·30	8·40	7·5	16·80	8·32	1·9
J.S.P.	1·65	0·30	—	1·95	15·4	15·60	7·76	5·2
W.L.	6·74	0·84	2·24	9·82	8·5	13·75	6·81	1·4
C.A.	7·02	1·47	2·90	11·39	12·9	15·95	7·90	0·13
O.D.B.	8·09	0·67	2·14	10·90	6·1	14·53	7·19	—
R.N.	6·38	0·54	2·40	9·32	5·8	13·05	6·46	0·14
A.G.P.	6·30	1·46	3·29	11·05	13·2	15·47	7·66	0·02
M.S.	6·65	1·12	1·25	9·02	12·4	12·63	6·25	—
I.S.	6·56	0·90	2·81	10·27	8·8	10·27	5·09	—
A.McF.	4·41	0·66	0·65	5·72	11·5	12·87	6·37	1·9
O.Q.	4·32	0·51	0·18	5·01	10·2	11·27	5·58	3·3

* The protein concentration values are obtained from the refraction increment values by means of the value 0·00202 [Schretter, 1926] for the specific refraction increment of total human serum protein.

Exp. 20. A series of normal human sera. We have next examined in turn the serum from a number of apparently healthy normal persons of both sexes. The results are collected in Table XVII. In all cases a 2·0 mm. cell was used and the general experimental conditions were very similar to those in the previously recorded experiments. The general shape of the corresponding curves in all these experiments coincides remarkably, and the curve in Fig. 14 may be taken as characteristic of the curves obtained at about the middle of each run. As we shall show in a subsequent paper the similarity of these curves of normal serum among themselves is quite sufficient to distinguish any of them at a glance from the curves which are obtained from many pathological sera. We regard a curve of the general shape shown in Fig. 14, with due consideration of the conditions of dilution, centrifugal force and time at which it was obtained, as a valuable physico-chemical criterion of the state of the proteins in a normal human serum. In the table of results it will be seen that while the calculated total protein concentration of the untreated serum has varied within wide limits (5·09 g./100 ml. (I.S.) to 8·43 g./100 ml. (J.S.P.)) the proportion of *G* fraction in all cases can be represented by the formula (10·5 ± 5) % of the total protein. The concentration of *X* fraction is (25 ± 5) % of the total protein, if we exclude the solutions diluted with more than an equal volume of 1 % sodium chloride. In the case of the latter solutions, owing to the increased boundary spreading, the *X* concentration values are very inaccurate, and indeed, must be regarded as little more than an empirical allowance for the small area between the *A* and *G* curves which cannot be included with graphical symmetry in either of these curves. It is probable that the true *X* values are greater than the values given.

The spreading coefficient and sedimentation constant values in Table XVII call for little comment as they are in the main what one would expect to find under the conditions of the various experiments. In certain cases no value is given for the sedimentation constant of the *X* molecules because the separation of the *X* curve was not sufficient to enable a sufficiently accurate location of the position of the "ideal" sedimentation boundary. Within the limits of comparability it appears from these results that the sedimentation constants of the fractions in human serum are the same as those of the corresponding fractions in cow and horse serum.

We have repeatedly obtained an irregular appearance of the *G* curve which will be seen on close consideration of the curve in Fig. 14. It is not clear to what extent this is due to the accentuation of the effect of base-line irregularities in relation to the small *G* curve, but we are of opinion on the whole that the *G* fraction in normal human serum is not homogeneous.

In only two cases in this series have we dialysed the serum beforehand and measured the total protein refraction increment, because we desired to examine the sera in as fresh and untreated a state as possible. The sera subjected to preliminary dialysis were those of C.A. and W.L., and the calculated values of the protein refraction increments were found to be respectively 7·5 and 14 % less than the measured values.

Another feature which we have noted is the occasional presence in human serum of quantities up to 5 % of the total protein of fractions which sediment between two and three times as fast as globulin and can usually only be seen in the first photograph. The molecular weight of these substances must be at least half a million. We have ignored these fractions in this work because they could only be satisfactorily studied with lower centrifugal forces than we wished to use. We mention their presence, however, because they must contribute in some degree to the discrepancies found between calculated and measured protein refraction increment.

The considerable disagreement among the three calculated values for the total protein refraction increment in the case of J.S.P. shows a phenomenon which we have found generally throughout this work, *viz.* much greater discrepancies between measured and calculated protein refraction increment values in the case of dilute solutions than of concentrated solutions. The explanation of this is not clear.

Exp. 21. The serum of mother and child. It is well known that the serum of a newborn infant contains smaller amounts of protein than the mother's serum. It occurred to us that the small amount of globulin present in normal adult serum might be associated with the development of immunity to various diseases acquired in the early years of life. We have therefore examined in the centrifuge serum from umbilical blood in the newborn infant, and serum from the mother's blood taken at the time of parturition. Owing to the lower concentration of total protein in the former serum we examined this without dilution. The same cell was used in both experiments and the other experimental conditions were comparable in the two cases. The results are shown in Table XVII opposite the reference letters I.S. (infant's serum), and M.S. (mother's serum). It is clear that our original idea is disproved since the infant's serum contains 8·8 % of a *G* fraction. The mother's serum has an abnormally low concentration of *X* fraction, the proportion being approximately half that in the infant's serum.

Exp. 22. A test of the reproducibility of a particular sedimentation diagram. The experiment which we mention here was actually carried out at the beginning of this work. After we had examined the writer's serum (*cf.* Table XVII), diluted 1:1·25 with 1 % sodium chloride, and discovered the surprisingly low concentration of globulin in it, our first concern was to repeat the finding. Another portion of the same diluted serum was, therefore, examined again a few days later using the same cell and approximately the same centrifugal force. The photographs were taken as far as possible at the same time in relation to the start of the experiment. We do not reproduce the results because these do not show any appreciable difference from the results of the earlier experiment shown in Table XVII. We found that if the curves of corresponding photographs were superimposed they coincided in practically every detail. Even some irregularities on the base-line, which we had previously ignored, were faithfully reproduced in the later experiment.

Exp. 23. Normal human serum dried by the Hardy-Gardner method. Serum dried by simple evaporation does not usually redissolve well, a residue of insoluble protein remaining behind on redissolving the powder. Hardy and Gardner [1910] have used a method of drying with alcohol in the cold which yields a white lipoid-free powder which redissolves completely. Sørensen [1930] has subjected the redissolved powder to a variety of tests and comes to the conclusion that the procedure "hardly involves any real chemical splitting, but only a purification of the proteins from the contained substances soluble in ether and alcohol". Also "reconstituted plasma can still be made to clot and albumins may be recrystallised" [Hewitt, 1927].

In view of our previous findings with serum dried by simple evaporation we have thought it of value to submit redissolved serum proteins obtained by the Hardy-Gardner method to the rather searching analysis of the ultracentrifuge. We divided a normal human serum into two portions one of which was dialysed (during which process some dilution took place) and examined in the centrifuge after the refractive indices of protein solution and dialysate had been measured.

The other portion was subjected to all the steps in the Hardy-Gardner drying procedure as described and modified by Hewitt [1927]. In particular, great care was taken to keep the temperature low and by the use of a refrigerator, itself contained in a cold room at −4°, the whole procedure was carried out at −16°. The serum-alcohol mixture was allowed to stand at −16° for 2 hours, and the subsequent filtration and washing of the protein precipitate occupied 6 hours at the same temperature. As recommended by Hewitt we have further minimised the time of contact between the proteins and the alcohol by omitting to dry the powder *in vacuo* before extracting with ether.

The final powder was pure white and redissolved completely in water. The solution was then dialysed against 1 % sodium chloride and the total protein concentration was arranged to be approximately the same as that in which the original serum was examined. The same cell, scale distance and centrifugal force were used in the two experiments. The results are shown in Table XVIII and we reproduce in Fig. 17 two curves—one from the original serum obtained 40 mins. after reaching full speed, and one from the dried serum obtained 25 mins. after reaching full speed.

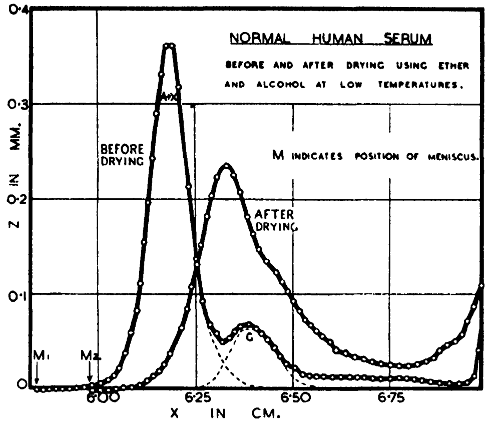

Fig. 17.

Table XVIII. *Results obtained with a diluted normal human serum and the same serum after drying with ether and alcohol.*

Cell thickness = 3·0 mm. Optical scale distance = 3·64 cm.
Mean centrifugal force = 255,000 times gravity.

	Average $S_{W,20}$		Average $(n_1 - n_0).10^3$			Measured total $(n_1 - n_0).10^3$ corr. to $\lambda = 366 m\mu$	"% diff." total $(n_1 - n_0)$	Calculated proportion of G mol.
	$A+X$ mol.	G mol.	$A+X$ mol.	G mol.	Total			
Original serum	3·9	5·70	5·42	0·56	5·98	7·31	−18·2	15·2
Dried serum	3·8	—	—	—	5·95	8·14	−26·9	—

It is clear that a marked change has taken place on drying. The solution of dried protein appears to contain considerable quantities of polydisperse protein, as shown by the irregularity of the curve and the discrepancy of 27 % between measured and calculated total protein refraction increment. We cannot say whether any unchanged native protein is still present or not. Signs of a *G* fraction can be discerned. It will be noted from Table XVIII that the average sedimentation constant of a boundary (taken as the position of the apex of the curve) in the case of the dried protein is in good agreement with the value for the same boundary in the case of untreated serum. It is possible that each fraction in the original serum has given rise to a group of polydisperse molecules with the same mean molecular weight as the parent protein fractions. If any of the original serum protein is still present in significant quantity we must conclude that the drying procedure has in some way given this native protein an abnormally high power of diffusion. We can imagine changes in the shape and charge of the protein molecules which might cause small alterations in the diffusion constant, but we cannot imagine a change which, without affecting the weight or homogeneity of the molecules in a fraction, could cause such a gross alteration in the degree of boundary spreading as we are concerned with here. The same phenomenon has been found in the case of a preparation of serum globulin dried in the same way.

SECTION 4. THE OSMOTIC PRESSURE OF UNDILUTED SERUM.

It has been noted by numerous observers [*e.g.* Mayrs, 1926; Verney, 1926; Krogh and Nakazawa, 1927] that when serum is diluted the colloid osmotic pressure falls more rapidly than corresponds to the fall in protein concentration. Various workers have attempted to correlate the osmotic pressure of a serum with the partial osmotic pressures of the albumin and globulin fractions in it [Govaerts, 1925; 1927; Marrack and Hewitt, 1927; Farkas, 1927; Adair and Robinson, 1930, 2; Elias and Goldstein, 1932], and it has been clearly established that a considerable proportion of the colloid osmotic pressure of a serum must be attributed to the uneven distribution of diffusible electrolytes across the membrane. The problem of determining accurately the proportion of the total pressure due to the diffusible electrolytes is one of great difficulty because we are concerned with a non-ideal solution. Adair and Robinson [1930, 2] in a careful consideration of the problem have come to the conclusion that Donnan's formulae, which refer to infinitely dilute solutions, are not applicable to serum as suggested by Hecht [1925] and Marrack and Hewitt [1927]. They put forward a new treatment of the problem which is essentially based on the calculation of the partial pressure of the diffusible ions from the membrane potential by means of the equation

$$p_i = 15{\cdot}4\,JE_m^2,$$

where p_i = the partial osmotic pressure due to the excess of diffusible ions inside the membrane;

J = the ionic strength, or the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valencies;

E_m = the membrane potential in millivolts.

Adair and Robinson have obtained good agreement between the observed osmotic pressure of dilute unfractionated serum proteins and the partial osmotic pressures of the albumin and globulin. They, therefore, suggest that the state of aggregation of these purified proteins is the same as in the diluted parent serum, a view with which the ultracentrifuge results are in agreement. They have also found that, if due allowance is made for p_i in the case of concentrated ox serum proteins, the relationship between the partial protein osmotic pressure and the total protein concentration of the solution approximates to a straight line. This fact is also not in essential disagreement with our results. Adair and Robinson conclude from the two sets of experiments, however, that the "state of aggregation of the proteins in untreated serum appears to be the same as their state of aggregation in the purified proteins", and with this conclusion we cannot agree.

Adair and Robinson have not attempted to demonstrate quantitative agreement between the observed osmotic pressure of undiluted serum and the partial osmotic pressures of the constituent albumin and globulin. We are informed in a private communication that this omission was intentional because of lack of confidence in the existing methods of estimating the albumin and globulin fractions in serum (which, incidentally, we regard as well justified), and because the osmotic coefficient g_p (a factor representing the effects of the attractive and repulsive forces between the molecules and presumed to be constant), may not be constant in concentrated solutions.

We have endeavoured to apply Adair and Robinson's theory to the results of osmotic pressure measurements which were carried out on the cow and horse serum used in the experiments of Sections 1 and 2, and on a specimen of human serum from Section 3 (C.A., Table XVII) of this work. The serum in each case was dialysed until ionic equilibrium was obtained against approximately ten times its volume of 1 % sodium chloride, and the height of fluid column in the manometer measured. The osmotic pressures are corrected to mm. mercury at 0°. We have also subjected each serum to a standard method of anaylsis involving precipitation with ammonium sulphate and thus obtained values for the albumin and globulin concentrations in the serum. We have no confidence that these values represent exact albumin and globulin concentrations, but they are as good as any that can be obtained to our knowledge and sufficient for our present purpose.

Osmotic pressure of undiluted cow serum.

Observed osmotic pressure of undiluted cow serum = 22·3 mm. Hg
Total protein concentration (C) = 7·64 g./100 ml. solution
Corrected protein concentration (C_v) = 8·27 g./100 ml. solvent

By precipitation analysis the serum was found to contain 58 % albumin and 42 % globulin. Therefore

$$C_{v\ \mathrm{albumin}} = 4{\cdot}80 \text{ g./100 ml. solvent}$$

and

$$C_{v\ \mathrm{globulin}} = 3{\cdot}47 \text{ g./100 ml. solvent}$$

By reference to Adair and Robinson's curve [1930, 2, Fig. 5, p. 1885] showing the variation of total osmotic pressure $(p_i + p_p)$ of crystalline serum albumin with protein concentration we obtain the value 15·5 mm. for an albumin concentration of 4·80 g./100 ml. solvent. This value is made up of 10·5 mm. due to the protein and 5 mm. due to p_i. Similarly, from their curve in Fig. 6 (p. 1886) we obtain the value 3·3 mm. for the total osmotic pressure of a solution containing 3·47 g. globulin/100 ml. solvent. This is made up of 3·0 mm. due to the protein and 0·3 mm. due to p_i. We may not add the total values together because it may not be assumed that the total p_i of a mixture is equal to the sum of the p_i values for the individual components in separate solution. It is more probable that the membrane potentials are strictly additive, especially in view of the rather constant E_m/C_v values which Adair and Robinson obtain for ox serum protein over a range of concentrations. That is

$$E_{m\ \mathrm{mixture}} = E_{m\ \mathrm{albumin}} + E_{m\ \mathrm{globulin}}.$$

Squaring both sides and substituting from the equation given above

$$p_{i\,\text{mixture}} = p_{i\,\text{albumin}} + p_{i\,\text{globulin}} + 2\sqrt{p_{i\,\text{albumin}} \times p_{i\,\text{globulin}}},$$

p_i will, therefore, be greater in the mixture than the sum of the p_i values due to each protein in separate solution, and by an amount which will be significant when the two components contribute approximately equally to the total p_i of the mixture, and negligible when one contributes much more than the other.

In the present case, the osmotic pressure of the serum

$$= 15{\cdot}5 + 3{\cdot}3 + 2\sqrt{5 \times 0{\cdot}3} = 21{\cdot}3 \text{ mm.}$$

Alternatively, we may use the albumin and globulin concentration values obtained from the sedimentation diagrams, *viz.* 83 % albumin and 17 % globulin (*cf.* Exp. 1). These are found to correspond respectively to partial osmotic pressures of 24·5 mm. and 1·3 mm. In this case $p_{i\,\text{globulin}}$ is negligibly small, and, therefore,

$$\text{Osmotic pressure of cow serum} = 24{\cdot}5 + 1{\cdot}3 = 25{\cdot}8 \text{ mm.}$$

Osmotic pressure of undiluted horse serum.

Observed osmotic pressure of undiluted horse serum = 20·5 mm.

Total protein concentration = 7·25 g./100 ml. solution

$C_{v\,\text{total protein}}$ = 7·82 g./100 ml. solvent

By precipitation analysis the serum was found to contain 51 % albumin and 49 % globulin. Therefore

$$C_{v\,\text{albumin}} = 3{\cdot}99 \text{ g./100 ml. solvent}$$

and $$C_{v\,\text{globulin}} = 3{\cdot}83 \text{ g./100 ml. solvent}$$

Again from Adair and Robinson's curves we obtain the corresponding osmotic pressures, *viz.* (4 + 8·2) mm. and (3·6 + 0·1) mm., giving a total osmotic pressure for the serum of $15{\cdot}9 + 2\sqrt{0{\cdot}82} = 17{\cdot}7$ mm.

By ultracentrifugal analysis the same serum was found to contain 80 % albumin and 20 % globulin (*cf.* Exp. 12). In this case, therefore,

$$C_{v\,\text{albumin}} = 6{\cdot}26 \text{ g./100 ml. solvent}$$
$$C_{v\,\text{globulin}} = 1{\cdot}56 \text{ g./100 ml. solvent}$$

and the corresponding osmotic pressures are

Albumin = 21·5 mm.
Globulin = 1·5 mm.
and Total serum protein = 23·0 mm.

Osmotic pressure of undiluted human serum (*C.A.*).

Observed osmotic pressure of undiluted human serum = 30·8 mm.

Total protein concentration = 7·91 g./100 ml. solution

$C_{v\,\text{total protein}}$ = 8·59 g./100 ml. solvent

Precipitation analysis gave 74·5 % albumin and 25·5 % globulin. Therefore

$$C_{v\,\text{albumin}} = 6{\cdot}40 \text{ g./100 ml. solvent}$$

and $$C_{v\,\text{globulin}} = 2{\cdot}19 \text{ g./100 ml. solvent}$$

Corresponding osmotic pressure of albumin = 22·0 mm.
Corresponding osmotic pressure of globulin = 2·2 mm.
and Corresponding osmotic pressure of serum protein = 24·2 + 0·3 = 24·5 mm.

Ultracentrifugal analysis gave 87 % albumin and 13 % globulin. Therefore

$$C_{v\ \text{albumin}} = 7{\cdot}47 \text{ g./100 ml. solvent}$$

and

$$C_{v\ \text{globulin}} = 1{\cdot}12 \text{ g./100 ml. solvent}$$

Corresponding osmotic pressure of albumin = 2·8 mm.
Corresponding osmotic pressure of globulin = 1·1 mm.
and Corresponding osmotic pressure of serum protein = 29·1 mm.

The results are collected in Table XIX.

Table XIX. *Comparing the observed osmotic pressure of undiluted serum with the theoretical value based on the albumin/globulin ratio obtained by ultracentrifugal and precipitation analysis.*

		Theoretical osmotic pressure	
Undiluted serum	Observed osmotic pressure mm. Hg	Based on precipitation *A*/*G* ratio	Based on ultracentrifugal *A*/*G* ratio
Cow	22·3	21·3	25·8
Horse	20·5	17·7	23·0
Man	30·8	24·5	29·1

In the derivation of the ultracentrifugal figures we have classed the combined *A* and *X* fractions as albumin, since we are unable to introduce allowances for the molecular weight of the *X* fraction. If we had sufficient data to do so, the serum osmotic pressure values which are based on the ultracentrifugal albumin/globulin ratio would be somewhat lower.

Strictly, we should only utilise Adair and Robinson's curves to obtain values for the osmotic pressure of albumin and globulin in equilibrium with a buffer solution of p_H 7·4 and ionic strength (J) equal to 0·3466. We have measured our serum osmotic pressures at approximately the p_H of the untreated serum and against an approximately 1 % solution of sodium chloride. These two factors, however, can only introduce small errors in our results since Mayrs [1926] and Marrack and Hewitt [1927] find the osmotic pressure of serum proteins to be only slightly influenced by changes of p_H in the neighbourhood of neutrality, and the ionic strength of our dialysate is approximately 0·34.

As already mentioned it is not yet proved that Adair and Robinson's theory may be applied in its present form to undiluted serum. We have ventured to apply it here for two reasons. Firstly, we wish to show that if the theory is applicable to undiluted serum it appears to agree with the ultracentrifugal albumin/globulin ratio at least as well as with the ratio obtained by precipitation analysis; and, secondly, we wish to put forward the above figures in order to give some idea of the magnitude of the difference in the theoretical osmotic pressures of undiluted serum based on the two conflicting analytical values for the albumin/globulin ratio.

GENERAL DISCUSSION.

We hesitate to attempt to generalise on the basis of the experimental results which have been given in this paper, because it will be clear that little more than a start has been made in the investigation of a very wide field. We have endeavoured to place on record an exact account of these experiments on normal sera in the hope that they may form a basis for the accumulation in the future of the considerably greater amount of experimental material which is clearly necessary before we may try to establish a comprehensive definition of the molecular dimensional state of the proteins in a normal serum. The necessity of having a much greater amount of experimental material than is at present available arises not merely because the exact definition of the normal serum of a species can only be obtained by averaging the results of a large number of normal members of the group, but also because we are attempting an exact physico-chemical definition of a non-ideal protein solution, and practically no general principles, such as we are familiar with in the case of dilute ideal solutions, can be applied. It will have been noticed in the foregoing description of our experimental results that the majority of our deductions have had to be based on comparisons between experiments. Our values for the sedimentation constant, for instance, have only a relative significance because of the high viscosity effects in strong protein solutions. Similarly, the molecular homogeneity of the serum fractions can only be estimated in a relative way by comparison of boundary spreading coefficients. We have no theoretical knowledge of the value of the boundary spreading coefficient of a pure protein in the same circumstances. Finally, the absolute concentration values of the serum fractions are subject to inaccuracies because of the effect of the total protein concentration which varies from one serum to another. All these facts raise difficulties in the way of generalising from the results obtained. They do not mean that established generalisations in this field can never be obtained. We believe that all that is required is a great many more results, preferably obtained with rather more standardised conditions of the centrifuge experiment than we have been able to adopt in this work. We believe that in the near future many new facts will be added to those quoted in this paper and we shall, therefore, attempt to draw the results together in the form of a few general deductions in the hope that these may form a framework of theory in relation to which present facts may be more easily viewed as a whole, and future facts may be tested.

In general, we have found that the normal serum of the cow, horse and man is constituted in a similar manner of three molecular species which can be identified by ultracentrifugal analysis. The corresponding fractions in the three types of serum appear to have the same sedimentation constants.

The heaviest of the three fractions separates easily from the other two sediments with a speed approximately the same as that of horse serum globulin in corresponding concentration and is present in variable amount up to 20 % of the total protein. The concentration of this fraction in a range of normal human sera was found to be rather constant, *viz.* (10 ± 5) % of the total protein concentration, which varied widely. The homogeneity of this fraction is doubtful in the case of human serum, which is also distinguished from cow and horse sera by a tendency to contain small amounts of substances sedimenting 2–3 times as quickly as globulin. These substances have not been specially examined.

The two lighter fractions differ so little in average molecular weight that it is difficult under the present conditions of a centrifuge experiment to obtain a satisfactory separation of the boundaries. We have, however, obtained a good separation in the cases of a few human sera by diluting slightly, and in these cases the average concentration of the more rapidly sedimenting fraction is 24 % of the total protein. We have no knowledge of the chemical nature of this substance, and the sedimentation constant tells us little more than that the molecular weight is slightly greater than that of serum albumin and considerably less than that of serum globulin. The sedimentation constant of the lightest fraction corresponds approximately to that of recrystallised horse serum albumin in the same circumstances, and consideration of the degree of boundary spreading suggests that the fraction is homogeneous. The separation of these two fractions is not so satisfactory in the case of cow and horse serum, and we can only give approximate values for the relative concentrations of the fractions and the sedimentation constant of the heavier of the two. The concentration of the latter is 14 % of the total protein in the cow serum and 16 % of the total protein in the horse serum which we have examined.

All three types of serum on dilution with 1 % sodium chloride show an increased concentration of the globulin fraction with correspondingly diminished concentration of the lighter fractions. In these diluted sera it is impossible to discern a separation of the lighter fraction into two owing to the increased boundary spreading. Whereas the dilution effect is very marked with horse serum it is much less marked with cow and human sera. It is only necessary to dilute horse serum with 3 volumes of 1 % sodium chloride in order to raise the globulin proportion from 20 to 36 %. In the cow serum, on the other hand, 28 % appears to be the maximum globulin obtainable by dilution. Dilution of a human serum with an equal volume of 1 % sodium chloride had no significant effect on the globulin concentration, and dilution with 7 volumes of 1 % sodium chloride could only raise the proportion from 8·6 to 15 %.

It appears that in certain circumstances a solution of the serum proteins may contain a considerable amount of polydisperse protein molecules. We have found that drying cow serum by simple evaporation over P_2O_5, or drying and then ether-extracting the powder, or treating the serum with ammonium sulphate, all gave rise in varying degree to this polydisperse protein. We have also found that the discrepancy between measured and calculated protein values is always greater when we are examining dilute solutions, and this may also indicate the presence of polydisperse protein in these solutions. These facts suggest a considerable sensitivity on the part of the proteins to environmental changes. Svedberg and collaborators have shown in numerous investigations that changes of hydrogen ion concentration outside certain limits give rise to pronounced associative or dissociative changes, and these facts have frequently been confirmed by other investigators using different methods. Altogether, it would appear that in the examination of protein solutions only a few very restricted methods may be used and most careful standardisation of technique must be observed. We have found in this work that it is at least possible to dialyse serum against 1 % sodium chloride or acetate buffer at p_H 4·6 without affecting the molecular dimensional state of the proteins.

In addition to the production of some polydisperse protein, treatment with ammonium sulphate appears to cause an irreversible alteration in the concentrations of the two lighter fractions in serum. This fact, which will require further investigation, raises difficulties in the way of applying to untreated serum facts derived from a study of the properties of the fractions obtained by ammonium sulphate precipitation.

We have found that treating serum with alcohol at low temperatures, which is an integral step in a method of obtaining dry lipoid-free serum protein, gives rise to a marked change in the sedimentation diagram. It is probable that the major part of the protein exists in a polydisperse state, and the mean molecular weight of the fractions of this polydisperse protein which separate in the centrifugal field appears to be the same as that of the fractions in the parent serum. This latter fact explains why the redissolved powder in the hands of various investigators has shown physico-chemical properties very similar to those of the original serum. We find the phenomenon of interest not only because it indicates a serious defect in this technique of drying, but also because we visualise the change which takes place to be an early stage in the complex phenomenon of denaturation, and there would appear to be placed in our hands a convenient method of studying the latter, *viz.* by examining various proteins after treatment with alcohol at low temperatures.

In the investigation of the various problems dealt with in this paper we have, as already stated, obtained only the barest experimental data in many cases. We hope, however, that in the presentation of these data we have, at least, adequately demonstrated an important aspect of the ultracentrifuge, *viz.* as a unique analytical instrument capable of separating the components of a mixture in respect of their molecular dimensions and estimating with considerable accuracy the concentration of each.

SUMMARY.

1. The ultracentrifugal sedimentation diagram appears to describe a strictly reproducible physical property of a protein solution.

2. The sedimentation diagrams obtained for the serum of the normal cow, horse and man all show the presence of three molecular types characterised by their sedimentation constants. The sedimentation constants of corresponding molecular types in serum from different species are the same. The diagram appears to be a common qualitative characteristic of normal serum generally, such differences as exist between sera being of the nature of quantitative differences in the concentrations of the individual fractions and of the total protein.

3. Heat inactivation of cow serum at 56° has little or no effect on the sedimentation diagram.

4. The sedimentation diagrams in the sera examined are changed fundamentally by simple dilution, the change consisting always in an absolute increase in the concentration of the globulin fraction and a corresponding decrease in the concentrations of the two lighter fractions. In dilute solutions it is not possible to separate the two lighter fractions in the centrifugal field.

The dilution effect is marked in the case of horse serum and much less so in the case of cow and human serum. Slight dilution had no significant effect on the sedimentation diagram of human serum, and dilution with 7 volumes of 1 % sodium chloride only increased the globulin concentration from 8·6 to 15·4 % of the total protein.

5. Dialysis of cow serum against 1 % sodium chloride or acetate buffer does not affect the sedimentation diagram. Complete removal of electrolytes induces certain changes which may be due to charge effects.

6. If cow serum is dried by simple evaporation at reduced pressures, with or without ether-extraction of the dry powder, the redissolved protein gives rise to a solution which differs significantly from the original serum in containing considerable quantities of polydisperse protein. A similar irreversible formation of polydisperse protein is caused by treating the serum with ammonium sulphate.

7. There is evidence that, in addition, ammonium sulphate causes an irreversible increase in the concentration of the intermediate fraction in cow and horse serum at the expense of the lightest (or "albumin") fraction.

8. A study of the behaviour of the serum and plasma from the same horse shows that no characteristic fibrinogen molecule is present in the plasma, but the two show certain differences in the concentrations of the three characteristic protein fractions.

9. In the study of a range of normal human sera there has been found a very close similarity in the general shape of all the sedimentation curves, indicating an approximately constant concentration for the constituent fractions.

The concentration of the heaviest or globulin fractions throughout the series is (10 ± 5) % of the total protein concentration, which latter value varied from 5·09 g./100 ml. to 8·43 g./100 ml.

10. The theory that the small amount of the globulin fraction in normal serum may owe its presence to the acquired immunities of childhood is proved to be wrong by the finding that in the serum of a baby at the time of birth 8 % of this fraction was present. There are some points of difference between this diagram and that of the mother's serum taken at the same time.

11. Drying a normal human serum by a recognised procedure using ether and alcohol at −16° causes a fundamental change in the physical state of the proteins. The interpretation of this change is discussed.

12. Osmotic pressure measurements have been performed on certain of the sera used in this work, and the values obtained are considered in the light of a recent theory of the osmotic pressure of untreated serum.

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REFERENCES.

Adair and Robinson (1930, 1). *Biochem. J.* **24**, 993.
—— —— (1930, 2). *Biochem. J.* **24**, 1864.
Elias and Goldstein (1932). *Z. exp. Med.* **82**, 757.
Farkas (1927). *Z. exp. Med.* **53**, 666.
Govaerts (1925). *Compt. Rend. Soc. Biol.* **93**, 441.
—— (1927). *Bull. Acad. R. Med. Belg.* (5), **7**, 356.
Hardy and Gardner (1910). *J. Physiol.* **40**, 68.
Hecht (1925). *Biochem. Z.* **165**, 214.
Hewitt (1927). *Biochem. J.* **21**, 216.
Krogh and Nakazawa (1927). *Biochem. Z.* **188**, 241.
Linder, Lundsgaard and Van Slyke (1924). *J. Exp. Med.* **39**, 887.
Marrack and Hewitt (1927). *Biochem. J.* **21**, 1129.
Mayrs (1926). *Quart. J. Med.* **19**, 273.
McFarlane (1935). *Biochem. J.* **29**, 407.
Mutzenbecher (1933). *Biochem. Z.* **266**, 259.
Schretter (1926). *Biochem. Z.* **177**, 349.
Sørensen (1930). *Compt. Rend. Trav. Lab. Carlsberg,* **18**, No. 5.
Starlinger and Hartl (1925). *Biochem. Z.* **160**, 113.
Verney (1926). *J. Physiol.* **61**, 319.

LXXX. BLOOD PYRUVATE IN VITAMIN B_1 DEFICIENCY.

By ROBERT HENRY STEWART THOMPSON[1]
AND ROBERT EUGENE JOHNSON.

From the Department of Biochemistry, Oxford.

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In the course of recent work on the rôle of vitamin B_1 in the metabolism of brain, it was discovered [Peters and Sinclair, 1933] that lactate solutions in which avitaminous pigeon's brain cells had respired for 2 hours gave a positive nitroprusside reaction for pyruvic acid [Simon and Piaux, 1924]. This led to a quantitative investigation of the formation of pyruvic acid during the respiration of normal and avitaminous brain tissue *in vitro* [Peters and Thompson, 1934]. In the course of this work it was discovered that relatively large amounts of pyruvic acid appear during the respiration in lactate of the minced brain tissue from vitamin B_1-deficient pigeons, whereas only a negligible amount (too little to be detected by the nitroprusside reaction) appears during the respiration under similar conditions of the brain tissue from normal pigeons. Further indirect evidence was thereby obtained in support of the recent Embden-Meyerhof scheme, which includes pyruvic acid as a normal intermediary in the metabolism of carbohydrate in the animal organism [see Embden *et al.*, 1933; Meyerhof and Kiessling, 1933; Meyerhof, 1933].

But although it was shown that there was this marked difference in the rates of pyruvate formation during respiration *in vitro*, no significant difference between the contents of pyruvic acid of normal and avitaminous brains could be detected when the estimations were performed immediately after the death of the animal, that is to say, without any period of respiration *in vitro*. A possible explanation is that although avitaminous brain tissue does produce abnormally large amounts of pyruvic acid *in vivo*, this substance is not detectable owing to the fact that it largely diffuses out into the blood stream.

The pyruvic acid in the blood of normal and avitaminous pigeons and rats was therefore investigated. Our results show that there is a marked accumulation of pyruvic acid in the blood of both these animals when in a state of avitaminosis B_1. A preliminary account of this work has already been published [Thompson and Johnson, 1934].

METHOD.

The method employed has been firstly to investigate the bisulphite-binding capacity of trichloroacetic acid centrifugates of the blood of normal and avitaminous animals, which were being used in brain experiments. In order to determine the percentage of pyruvic acid in the total bisulphite-binding compounds present in the blood, parallel estimations were also made by isolating the 2:4-dinitrophenylhydrazone of pyruvic acid and estimating it colorimetrically by a modification of the Neuberg-Case method [see Case, 1932; Peters and Thompson, 1934].

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[1] Senior Demy of Magdalen College.

Determination of the bisulphite-binding capacity of pigeon's blood.

The experiments with pigeons were carried out as follows. The bird was first rested for one hour by being wrapped in a duster and placed in a dosing-box kept in the dark; we have found that by this method more uniform results are obtained than if the bird is taken straight from the cage. At the end of the hour the bird was taken out of the box and killed by guillotining. The blood was allowed to drain into a clean dry beaker, and 3 ml. were then rapidly measured with a clean dry pipette into a weighed 25 ml. centrifuge-tube containing 2 ml. of 25 % trichloroacetic acid and 5 ml. of distilled water. The exact amount of blood taken was obtained by re-weighing the tube. The mixture was then allowed to stand for half an hour, at the end of which time it was centrifuged and re-extracted twice with 5 ml. portions of 5 % trichloroacetic acid. The combined centrifugates were then brought approximately to p_H 2·0 with 40 % sodium hydroxide and made up to 25 ml. in a glass-stoppered volumetric flask.

10 ml. aliquots were taken, treated with sodium bisulphite and titrated with $N/100$ iodine for bisulphite-binding capacity by the method of Clift and Cook [1932]. In order to prevent the frothing which tends to occur on the addition of the sodium bicarbonate to hydrolyse the bisulphite compound, one drop of capryl alcohol was added in some of the experiments immediately before the addition of the bicarbonate; capryl alcohol used in this way has no bisulphite-binding capacity.

All but one of the avitaminous birds used had been dosed with glucose once, according to the usual procedure adopted in this laboratory, and all showed head-retraction at the time of use.

RESULTS.

The values obtained with pigeons are shown in Table I (see also Fig. 1). This table shows that there is a very large and constant increase in the bisulphite-binding capacity over the normal level in the blood of vitamin B_1-deficient pigeons.

Table I. *Bisulphite-binding substances in the blood of normal and avitaminous pigeons.*

mg. pyruvic acid per 100 g. blood (1 ml. $N/100$ I = 0·44 mg. pyruvic acid).

Normal		Avitaminous	
Exp.	B.B.S.	Exp.	B.B.S.
5	4·34	6	10·53
7	4·94	8	17·03
9	3·97	16	12·60
15	4·45	18	11·39
20	3·92	22	10·64
21	3·57	23	13·10
25	4·07	24	10·92
36	2·82	26	8·20
38	5·14	28	6·27
48	3·18	29	11·95
63	3·69	39	9·12
64	3·71	40	6·82
66	3·67	42	12·12
		59	17·70
Average	**3·96**	Average	**11·31**

In order to correlate this difference specifically with vitamin B_1-deficiency, estimations were also made on the blood of birds which had developed opisthotonus, but which had been cured by the administration of vitamin B_1 concentrates; in one experiment (Exp. 60) crystalline vitamin B_1 was used. No food was given after dosing, so that their condition of starvation remained strictly analogous to that of the avitaminous birds. The water allowed after dosing was limited to 5 ml. in order to control any possible "washing-out" of the pyruvic acid. The results obtained with the "cured" birds are shown in Table II. Mr H. W. Kinnersley dosed the birds for us. We should like to thank him at this point.

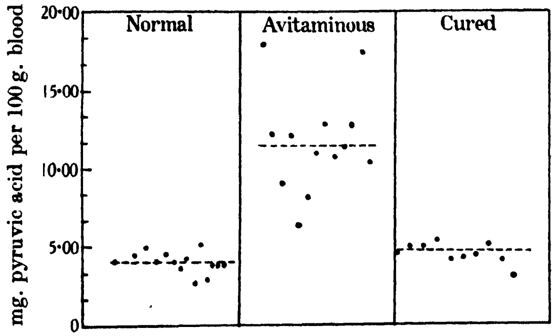

Fig. 1. Bisulphite-binding capacity of pigeon's blood. Broken lines through averages.

Table II. *Bisulphite-binding substances in the blood of "cured" birds.*

Results expressed as in Table I.

Exp.	B.B.S.	Condition
27	3·77	
30	4·73	
32	5·72	Bird still showed "leg weakness"
33	5·10	
34	4·89	
35	4·77	
37	7·03	"Leg weakness" and partial blindness still present; previously gave anomalous tests
53	6·12	
54	5·55	
60	5·25	Dosed with crystalline vitamin B_1.
Average	**5·29**	

These results show that the bisulphite-binding capacity of the blood returns to a figure only a little above the normal (and not statistically different from the normal) after the nervous symptoms have disappeared on dosing with vitamin B_1. This increase in the bisulphite-binding capacity of avitaminous blood is therefore a specific effect of the avitaminosis and is not merely the result of the accompanying starvation.

The results in Tables I and II have been examined statistically by means of Fisher's "t" test. The difference between the normal and avitaminous levels is certainly significant, whereas no real difference exists between the normal level and that found for the "cured" birds.

Determination of pyruvic acid as 2:4-dinitrophenylhydrazone.

Parallel estimations on pigeon's blood, made by a modification of the Neuberg-Case method [Peters and Thompson, 1934], showed that this increase in the bisulphite-binding capacity of the avitaminous bird's blood is probably due entirely to pyruvic acid. The results are shown in Table III. It was unnecessary to extract the blood of "cured" birds, because even if all the bisulphite-binding capacity of their blood were due to pyruvic acid, which is highly unlikely, the level of pyruvic acid in the avitaminous bird's blood would still be higher than that in the "cured" bird's blood.

Table III. *Amounts of pyruvic acid in the total bisulphite-binding substances in pigeon's blood.*

	mg. pyruvic acid, per 100 g. blood.		
Exp.	B.B.S.	Pyruvic acid	Non-pyruvic acid B.B.S.
	Normal.		
48	3·18	0·82	2·36
63	3·69	0·80	2·89
64	3·71	0·90	2·81
	Avitaminous.		
39	9·12	6·22	2·90
40	6·82	4·38	2·44
26	8·20	6·34	1·86

For these estimations the blood centrifugates, at p_H 2·0, were made up to 25 ml.; 5 ml. aliquots were then taken for bisulphite estimations, the remaining 15 ml. being used for 2:4-dinitrophenylhydrazine extractions. Only six experiments of this type were done because of the unequivocal results.

Identification of pyruvic acid. The blood centrifugates were first tested by the nitroprusside reaction of Simon and Piaux [1924]. Normal pigeon's blood gave a centrifugate showing a faint and evanescent red colour, probably due to sulphhydryl groups. Avitaminous bird's blood showed a slowly developing green colour; in one case the blue of pyruvic acid in fairly large concentrations was seen.

For more certain identification, however, the 2:4-dinitrophenylhydrazone of pyruvic acid was extracted and purified as follows.

Trichloroacetic acid centrifugates were collected from the blood of fifteen avitaminous pigeons. The mixed centrifugates were kept in strongly acid solution in the presence of 2:4-dinitrophenylhydrazine for 24 hours. The mixture of hydrazine and hydrazones was then taken up in ethyl acetate. The ethyl acetate solution was repeatedly shaken with alkaline sodium phosphate solution until no more of the pyruvic acid 2:4-dinitrophenylhydrazone came out. The phosphate solution was then acidified with hydrochloric acid, and the mixture of hydrazones with an accompanying impurity of hydrazine was once more brought into ethyl acetate solution. The whole procedure was repeated until the alkaline phosphate removed the last trace of yellow from the ethyl acetate phase. The ethyl acetate phase was then collected and evaporated to dryness *in vacuo* at 15°. The residue was extracted with light petroleum, and the yellow hydrazone left undissolved was taken up in ethyl acetate and purified by solution in sodium phosphate, acidification with HCl and re-extraction with ethyl acetate. This was repeated five times. The final ethyl acetate solution was washed with distilled water brought to p_H 2·0 with HCl, and the ethyl acetate phase was then drawn off and evaporated *in vacuo* at 15° to a small volume. The hydrazone was precipitated with excess light petroleum and was dried *in vacuo* at about 15° over sulphuric acid.

The final yellow product had the following characteristics:

(1) It gave a red colour with alcoholic potash identical with that given by synthetic pyruvic acid 2:4-dinitrophenylhydrazone.

(2) It melted variously at 210°, 216°, 214°, 220°, 216°, 214° (corrected). The melting-point of pyruvic acid 2:4-dinitrophenylhydrazone is given as 214° [Allen, 1930; Case, 1932]; 216° [Neuberg and Kobel, 1929]; and 219° [Lohmann and Meyerhof, 1934]. We have noticed that different crystals of our purest products melt at different temperatures from 214–220°.

(3) Mixed with synthetic pyruvic acid 2:4-dinitrophenylhydrazone, it melted at 213°, 219°, 216° (corrected). Average 216°.

Enough hydrazone has not yet been collected for a satisfactory analysis, but this is now being carried out. We feel however that there is little doubt that the compound responsible for the rise in the bisulphite-binding capacity of polyneuritic pigeon's blood above normal is pyruvic acid, or some labile compound of pyruvic acid which yields free pyruvic acid in strongly acid or alkaline solution. Phosphopyruvic acid [Lohmann and Meyerhof, 1934] would give the same results as we have found under the conditions of our experiments.

EXPERIMENTS WITH RATS.

The bisulphite-binding capacity of the blood of normal rats and of rats in acute avitaminosis B_1 was determined in essentially the same way as for pigeons.[1] The rats however were taken straight from the cage, without any preliminary period of resting, and the blood was drained from the neck directly into a weighed 50 ml. centrifuge-tube containing the usual amount of trichloroacetic acid. *N*/200 iodine was used for the titrations instead of *N*/100. The results are shown in Table IV.

Table IV. *Bisulphite-binding substances in the blood of normal and avitaminous rats.*

mg. pyruvic acid per 100 g. blood.

Normal		Avitaminous	
Exp.	B.B.S.	Exp.	B.B.S.
95	3·28	75	11·67
96	3·82	76	7·13
129	4·82	93	11·61
130	4·95	94	9·18
		97	11·21
		120	6·81
		123	8·13
Average	**4·22**	Average	**9·39**

These results are in complete agreement with those obtained with pigeon's blood. Hydrazone extractions have not been done in the case of rat's blood, but we have found that in every case the solutions from the avitaminous rats gave the green nitroprusside reaction characteristic of pyruvic acid in low concentration, while the solutions from normal blood gave only the evanescent pink reaction of sulphhydryl compounds.

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[1] We thank Mr J. R. P. O'Brien for supplying the rats used.

DISCUSSION.

The results presented in this paper interested us primarily in view of the possible interpretation which they provided for the previous results obtained by Peters and Thompson [1934] with the brain. It is possible that the amounts of brain used by these workers were too small to allow the detection of the increased pyruvic acid in avitaminosis B_1 without a period of respiration *in vitro* to allow for its accumulation, but the raised blood pyruvate suggests that there is also a diffusion of pyruvic acid out into the blood stream.

There now arises the question of whether this raised blood pyruvate is due to metabolic disturbances in the brain alone, or whether it is a reflection of a general lesion throughout the tissues. In view of the work of Fisher [1931] on the lactic acid metabolism of various tissues in avitaminosis B_1, it seems unlikely that the brain alone is responsible for so large an increase in the level of this acid in the blood. In this connection it is also of interest that one of us (R.H.S.T.) found a powerful nitroprusside reaction for pyruvate in lactate solutions in which avitaminous kidney tissue had respired for two hours, whereas the solution in which the normal tissue had respired showed only a very faint colour. Moreover, Embden *et al.* [1933] have shown that pyruvic acid is a normal intermediary in the carbohydrate metabolism of muscle, and Elliott and Schroeder [1934] showed that kidney slices are capable of oxidising pyruvic acid about twice as fast as lactic acid. It seems likely therefore that there is a general lesion throughout the tissues in avitaminosis B_1 resulting in increased blood pyruvate, although we have not attempted to correlate the rise in blood pyruvate with the onset of symptoms.

We feel that the work of Rüter [1923] and of Bornstein and Ascher [1926] excludes any possible interference with our results for avian blood by glycolysis *in vitro*; it was shown by these workers that glycolysis in avian blood occurs very slowly in the absence of inhibitors of cellular respiration. We have ourselves tested this point by allowing the pigeon's blood to drain directly into the trichloroacetic acid and have found that the values obtained did not differ from those obtained when the blood stood for 30–40 seconds before being mixed with trichloroacetic acid. In the experiments with rats the blood has always been drained directly into the protein precipitant.

In view of the possible clinical application of this work as a test for vitamin B_1 deficiency, we are investigating the bisulphite-binding capacity of human blood.

SUMMARY.

1. The bisulphite-binding capacity of the blood of normal and B_1-avitaminous pigeons and rats has been investigated.

2. There are abnormally large amounts of bisulphite-binding substances in the blood of B_1-avitaminous pigeons and rats, an accumulation independent of the accompanying starvation.

3. This accumulation is specifically related to the vitamin B_1 deficiency, and not to the accompanying starvation, because the blood of pigeons cured by vitamin B_1 of the acute symptoms of avitaminosis has a normal level.

4. The increase in bisulphite-binding capacity of the blood of avitaminous pigeons is probably due entirely to pyruvic acid.

We should like to express our gratitude to Prof. R. A. Peters for his encouragement and advice during this research, to Mr R. B. Fisher for the statistical examination of our result and to the Medical Research Council for a grant towards expenses.

REFERENCES.

Allen (1930). *J. Amer. Chem. Soc.* **52**, 2955.
Bornstein and Ascher (1926). *Z. ges. exp. Med.* **52**, 607.
Case (1932). *Biochem. J.* **26**, 753.
Clift and Cook (1932). *Biochem. J.* **26**, 1788.
Elliott and Schroeder (1934). *Biochem. J.* **28**, 1920.
Embden, Deuticke and Kraft (1933). *Klin. Woch.* **12**, 343.
Fisher (1931). *Biochem. J.* **25**, 1410.
Lohmann and Meyerhof (1934). *Biochem. Z.* **273**, 60.
Meyerhof (1933). *Nature*, **132**, 337, 373.
—— and Kiessling (1933). *Biochem. Z.* **264**, 40.
Neuberg and Kobel (1929). *Biochem. Z.* **216**, 495.
Peters and Sinclair (1933). *Biochem. J.* **27**, 1910.
—— and Thompson (1934). *Biochem. J.* **28**, 916.
Rüter (1923). *Z. ges. exp. Med.* **37**, 151.
Simon and Piaux (1924). *Bull. Soc. Chim. Biol.* **6**, 477.
Thompson and Johnson (1934). *Chem. Ind.* **53**, 1024.

LXXXI. CRYSTALLINE VITAMIN B_1.

By HENRY WULFF KINNERSLEY, JOHN RICHARD O'BRIEN AND RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

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Crystals with vitamin B_1 activity isolated from baker's yeast by Kinnersley *et al.* [1932; 1933] differed from those of others in certain respects. Further work failed to resolve differences [Kinnersley *et al.*, 1934]. But lately, co-operation with Prof. Windaus and collaboration with Dr Holiday [Holiday *et al.*, 1934] have led to a revision of some of the facts published from other laboratories, which eliminates the main differences between ourselves and them. Valuable help has also been obtained from X-ray analysis methods. So that in our opinion, there is now no valid reason for questioning the view that vitamin B_1 has been isolated, though certain details have not yet been settled.

Historical.

Jansen and Donath [1926] were the first to isolate crystalline vitamin B_1 preparations. These were not quite pure; they contained substances precipitable by mercuric sulphate in acid solution, which is not a property of the vitamin [Kinnersley and Peters, 1928; Van Veen, 1932]. Much later, Ohdake [1932]; Van Veen [1933]; Windaus *et al.* [1932]; ourselves [1934]; Williams *et al.* [1934] obtained crystals, which were claimed to be of higher activity; this issue of activity was however somewhat blurred. Windaus *et al.* [1932] stated that their crystals reached an activity (pigeon dose) of 2·4γ per diem under the conditions of their tests (injection of 6γ). Later it was concluded by Van Veen that the Windaus preparation had the same activity as that of Jansen and Donath [1926]. Since the activity of a sample of this latter had been determined previously in this laboratory [Jansen *et al.*, 1930], and had been found to be less active, there were grounds for thinking that our own preparations actually had a higher potency. Such a view was confirmed by a parallel test upon some crystals (Windaus 1) sent by Prof. Windaus and some of our own; ours proved more active than theirs. The difference between the two crystalline samples was also found to persist for two other new methods of test devised in this laboratory, namely the catatorulin test upon brain enzyme systems [Passmore *et al.*, 1933] and the formaldehyde azo-colour test [Kinnersley and Peters, 1934]. In the meanwhile Seidell and Smith [1933] reported for our crystals as tested upon rats an activity of 80γ as against 120γ for a sample from Windaus. They did not consider the difference to be significant. Van Veen [1934] however reported that our crystals were more potent; and later he claimed to have made a preparation of higher activity than ours. We hesitated to accept this because he mentioned that his most active sample contained 500 units of vitamin B_1 per mg. which was very little different from our estimates. In fact a new specimen of his vitamin B_1 was not distinguishable from ours by animal test, though this specimen might have become less active in transit. Our conclusion had to be that there was no difference between our preparation and that of Van Veen, but that the Windaus 1 sample was not the same as these two.

The differences between the products of this laboratory and those of others were reflected in other attributes of the crystals. All seemed to be agreed that the formula was either $C_{12}H_{18}O_2N_4S$ or $C_{12}H_{16}ON_4S$ for the base. Bernal and Crowfoot [1933] stated in a report on the X-ray analysis that the crystals from different laboratories were the same. On the other hand we found slightly different analytical figures, in particular a C content of 42·2 against 40·6 %, and slightly higher S and Cl values. There was also a marked difference in the ultraviolet absorption spectra; the band reported by Windaus *et al.* was at 260 $m\mu$ in alcoholic solution, whereas that of ours was at 247–249 $m\mu$ [Peters and Philpot, 1933]; (see Holiday [1935] for a discussion of this point).

Convinced that our facts were correct, and that these differences were therefore real, we searched for a more active vitamin component. It was found possible to refractionate the vitamin crystals with phosphotungstate, which has proved a powerful method of attack at earlier stages. By collection of the precipitates at different p_H values however, no certainly more potent sample was reached.

Fractionation experiments with phosphotungstate were carried out by taking the crystals and refractionating within narrow limits. In the later experiments formalin was added. Though the question has not been properly investigated there is some evidence in our hands that formalin shifts the points of precipitation of some bases more than others; in this case it should prove a refinement of phosphotungstate fractionation. Theoretically, it should influence the point of precipitation by interfering with the basicity of a group such as —NH [*cf.* Barnes and Peters, 1932]. In its presence vitamin B_1 is stable provided that the p_H is not made greater than 6·0; the precipitation point is shifted towards a more acid reaction. The claim by Malcolm [1933] that formaldehyde destroys vitamin B_1 cannot readily be reconciled with our observations. A typical formalin experiment is as follows:

Exp. 1. A solution of about 50 mg. of the vitamin B_1 crystals in water was adjusted to p_H 7 and an equal volume of 40 % formalin added—volume 35 ml. 1·6 ml. of 10 % 24-phosphotungstic acid were added. The p_H was now 5·6–6. The precipitate was collected and washed with $N/10$ sulphuric acid and the washings were added to the filtrate. The precipitate was ground with solid baryta in the usual way and worked up into the crystals. By the addition of 1·0–1·5 ml. $N/10$ sulphuric acid and phosphotungstic acid as required, precipitates were collected in order at about p_H 4 A, B, C and D, and crystals were prepared from each fraction. The p_H was not determined exactly, the aim being to collect what appeared to be zonal precipitations. The total volumes used in the precipitation were 6 ml. $N/10$ sulphuric acid and 3 ml. 10 % sodium phosphotungstate. The precipitates C and D were too small for analysis. For A and B, we have (10γ doses given):

	Wt. of crystals recovered (mg.)	Birds	Day-dose γ
A	11·4	12	2·17
B	17·5	16	2·00

The colour reactions were of equal intensity. There is no evidence of increased activity in either A or B which might have been expected if the crystals were a mixture.

Procedures which would inactivate the vitamin were not valid, and benzoylation is eliminated by the well-known fact that the vitamin escapes this treatment [Seidell, 1929]. Attempts to fractionate by use of lead acetate also gave no success. Since such a procedure separates some nucleotides, substances of pyrimidine type might have been thus removed. More success attended the preparation of the nitrate and the sulphate, which gave opportunity for further tests and for observation by X-ray analysis. No more active vitamin was however reached.

At this stage, Dr Holiday reinvestigated the ultraviolet absorption curve of some of our sulphate and hydrochloride and also of the hydrochloride of Van Veen. The results in acid alcoholic solution reproduced those obtained earlier by Peters and Philpot. A second sample from Prof. Windaus (Windaus 2), which he stated to have been many times recrystallised as the chloroaurate, showed the same spectrum and molecular extinction coefficient as our own. Holiday [1935] has also found that the spectrum of vitamin B_1 changes profoundly according to the conditions of solution and p_H.

This accounts for puzzling points in the curves of Heyroth and Loufbarrow [1934]. It seems clear that all active specimens of vitamin B_1 have the same absorption band with the peak at 247–249 $m\mu$ when tested in acid alcohol solution. Preparation 2 of Windaus showed the same biological activity, catatorulin activity and intensity of formaldehyde-azo-reaction as our own; hence biological differences have disappeared. Furthermore in private communication Prof. Windaus has told us that intensive drying in high vacuum at 80° raises his values for C content to 42·2 %[1], in substantial agreement with our own which were obtained from material dried in a vacuum desiccator; and his values for absorption bands are now in agreement with ours. There are therefore no longer grounds for a belief that the purest crystalline preparations of vitamin B_1 from yeast and rice polishings are substantially different. Since we have been unable to prepare a more potent fraction in spite of strenuous endeavour, we must conclude that the crystals are actually vitamin B_1.

Though the problem as to identity of the crystals with vitamin B_1 may be considered to be settled, we do not feel that all questions connected with the formula are satisfactorily answered; we propose to give our data pointing out discrepancies. Doubtless, determination of the vitamin constitution by other methods will lead to their resolution.

Preparation of salts. The hydrochloride was prepared as before; nitrates and sulphates as follows. The sulphates prepared have the approximate composition $2X(SO_4)_3$ low M.P. and $2X(SO_4)_4$ high M.P. (X = base).

Vitamin B_1 sulphate. On converting the hydrochloride into the sulphate, two compounds are obtained with melting-points of 203°[2] and 276 to 278° respectively. The salt of low M.P. is obtained at p_H 3, that of high M.P. at p_H 1.

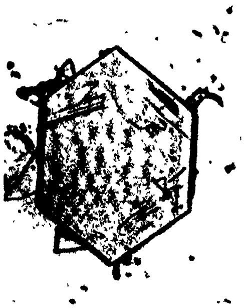

Fig. 1. Sulphate of low M.P. ×200.

Low M.P. *sulphate* (Fig. 1). To 20 mg. vitamin B_1. HCl a hot saturated solution of Ag_2SO_4 is added until a precipitate of AgCl is no longer obtained (about 2 ml.). Absolute alcoholic H_2SO_4 (p_H 3) is added, the precipitate centrifuged off and washed with 1 or 2 drops of water and absolute alcoholic H_2SO_4 (p_H 3)[3]. The filtrate is concentrated on the water-bath, first in a glass evaporating basin and then in a test-tube, and any inactive precipitate obtained is centrifuged off. Addition of absolute alcohol is made from time to time until most of the water has been removed and absolute alcoholic H_2SO_4 is added if necessary.

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[1] It is to be noted that some of the analyses of Jansen *et al.* and Ohdake show figures for C content higher than 40·6 %.

[2] Values for M.P. corrected throughout.

[3] Prepared by adding 1–2 drops concentrated H_2SO_4 to 50 ml. redistilled absolute alcohol (not dried) so that a drop tested on a white plate with bromophenol blue shows approximately the colour for p_H 3·0.

A solution is finally obtained which inclines to be opaque and from which a precipitate settles out on cooling while it redissolves on warming. If the solution is left overnight in the cold chamber, there is obtained a clear solution with sulphate crystals round the sides of the test-tube.

Sulphate of high M.P. To 20 mg. vitamin B_1.HCl a drop of N H_2SO_4 is added before decomposing the hydrochloride with Ag_2SO_4. It is then worked up as above. The addition of N H_2SO_4 to p_H 1 is only made in the last stages in order to avoid the risk of inactivation by concentrating at this acid p_H.

Nitrate of vitamin B_1 crystals. To 10 mg. vitamin B_1.HCl is added 1 ml. 1 % $AgNO_3$ (theory: no excess); it is warmed upon the water-bath, 4 ml. absolute alcoholic HNO_3, p_H 2, are added, the solution is centrifuged and the precipitate of AgCl washed with 1 drop of water and absolute alcohol.

The filtrate is then concentrated on the water-bath to remove most of the water and worked into about 97 % alcohol at p_H 3 in the usual way. A yellow inactive precipitate which comes out is discarded. Yield 10·7 mg.

Melting-points. Various observers state that vitamin B_1 hydrochloride melts with decomposition; Windaus *et al.* [1932], 250° "unter Zersetzung"; Ohdake [1934], 249° (uncorrected) with decomposition; Van Veen [1933], 250° (corrected); Williams *et al.* [1934], 249–250°.

In spite of much attention to this point, we have never obtained melting-points as high as 250° for our hydrochlorides. We usually determine M.P. in small open tubes in Mason's melting-point apparatus under microscopic observation; we have satisfied ourselves that these results do not differ from those in sealed tubes. The M.P. for the hydrochloride is not sharp and is usually 230 ± 1–$2°$. It makes little difference whether the compound is heated up slowly or quickly to a temperature of 200–220°. It is best to allow the temperature to rise very slowly for the last 5°, taking 3 or 4 mins. for this period. After a definite change at about 230°, no further alteration is noticed up to 270°. In marked contrast to the hydrochloride the sulphates melt sharply, that of lower M.P. at 203°, and that of the higher M.P. at 274–276°. The nitrate decomposes sharply at 164–165°.

The empirical formula of vitamin B_1.

For the hydrochloride Windaus *et al.* [1932] proposed two alternative formulae (Nos. 1 and 2, Table I) differing by H_2O. Their hydrochloride agreed with No. 1, and their picrolonate with No. 2. Ohdake [1934] considers that the formula is $C_{12}H_{16}O_2N_4S.2HCl$, Van Veen $C_{12}H_{20}O_2N_4S.2HCl$, and Jansen *et al.* [1933] support the Windaus formula No. 1.

Our own analyses give as average the following (see Appendix I):

	C	H	N	S	Cl	
Hydrochloride	42·2	5·7	15·1	9·5	20·9	(Schoeller) previous value with addition of one further S analysis =9·42 %
	42·1	6·2	15·6	9·75	20·94	(Kinnersley and O'Brien, see Appendix I)

Sulphate	C	H	N	S	SO_4
High M.P.	30·33	5·8	12·33	18·11	40·1
Low M.P.	35·00	5·50	13·38	18·51	34·7

The possible formulae based upon $C_{12\pm1}$ are calculated in Table I.

Table I. *Possible formulae for vitamin B_1 hydrochloride.*

No.	Formula	C	H	N	S	Cl	Total mol. wt.	Base only
1	$C_{12}H_{18}O_2N_4S.2HCl$	40·6	5·7	15·8	9·0	20·0	355	282
2	$C_{12}H_{16}ON_4S.2HCl$	42·7	5·34	16·6	9·5	21·05	337	264
3	$C_{12}H_{18}ON_4S.2HCl$	42·45	5·9	16·5	9·44	20·92	339	266
4	$C_{24}H_{34}O_3N_8S_2.4HCl$	41·6	5·5	16·2	9·25	20·5	346*	273*
5	$C_{24}H_{36}O_3N_8S_2.4HCl$	41·4	5·76	16·1	9·24	20·45	347*	274*
6	$C_{24}H_{36}O_3N_7S_2.4HCl$	42·4	5·9	14·4	9·43	20·9	340*	267*
7	$C_{24}H_{34}O_3N_7S_2.4HCl$	42·5	5·61	14·45	9·46	20·95	339*	266*
8	$C_{24}H_{34}O_4N_7S_2.4HCl$	41·5	5·48	14·1	9·22	20·45	347*	274*
8*a*	$C_{24}H_{32}O_2N_7S_2.4HCl$	43·6	5·46	14·85	9·7	21·5	330*	257*
9	$C_{23}H_{34}O_2N_7S_2.4HCl$	42·4	5·84	15·1	9·85	21·8	325*	252*
10	$C_{25}H_{36}O_4N_7S_2.4HCl$	42·4	5·65	13·8	9·04	20·1	354*	281*
11	$C_{25}H_{36}O_3N_8S_2.4HCl$	42·5	5·66	15·85	9·08	20·1	353*	280*

= half mol. wt. values.

The most satisfactory fit for the analytical figures is evidently formula 9, but this must be excluded upon the results of X-ray analysis.

By crystallographic methods Bernal and Crowfoot [1934] obtain the values given in Table II; double the values given are not excluded by X-ray analysis. The value for the hydrochloride is the most accurate.

Table II. *Possible mol. wt. (lowest formula wt. for base).*

	Min.	Prob.	Max.
Hydrochloride	270	278	286
Sulphate* (low M.P.)	249	270	286
Nitrate*	263	274	286

* Personal communication.

Values below 263 are certainly excluded and below 270 doubtful. Hence 8*a* and 9 are eliminated. 1, 4, 5, 8 can be excluded upon C, Cl, S values, 10, 11 upon Cl, S values. The mol. wt. is not consistent with 8*a*, 9, or really with 2, 3, 6, 7.

No formula therefore agrees with the analyses and mol. wt. estimations by X-ray analysis. If we consider that the X-ray analysis is some 2 % in error, then formulae 2, 3, 6 and 7 are possible. The nitrogen determinations fit none of these, being intermediate in value. There is some fundamental difficulty about nitrogen estimation in the hydrochloride to which Windaus *et al.* [1932] have already drawn attention. Jansen *et al.* [1933] obtained values nearer 16 % by treating with potassium chlorate. For the sulphates, evidently the difficulty does not arise. It should be mentioned that the nitrogen analyses in the laboratory were controlled by estimations of known substances, such as sulphanilic acid, which gave theoretical results under the same conditions within an error of 0·5–1 %

In our view, the high C values mean that No. 1, the formula proposed by Windaus *et al.* and supported by the others mentioned, is not the simplest vitamin B_1 formula. We favour the second of their proposals Nos. 2 or 3, as a working hypothesis. But in doing so, it must be recognised that there are still unreconciled data. Possibly it is not water but —OH which is the difference between the two formulae; in this case the vitamin would exist in two states of oxidation, and act as an —OH donator. We are not inclined to favour the idea that the vitamin exists as an N_3 compound as well as N_4. This idea could be reconciled with the mol. wt. but not with the nitrogen determinations [*cf.* Heyroth and Loufbarrow, 1934]. A mixture however would explain these.

Biological activity.

The curative pigeon day dose method with injection of vitamin has been used in the majority of tests; it is not very satisfactory for the crystalline vitamin. The relation between dose and time of cure has broken down in some cases. In Table III are given the average values for injection of 10γ. The final values reached are controlled (*a*) by an integration of tests made throughout the year to avoid seasonal changes and (*b*) by simultaneous tests; 92 A 50, 34 A 3 for instance in July 1934 were made by the method of alternate dosing; these give results near the average.

Table III. *Curative pigeon test with vitamin B_1 salts.*

10γ doses injected.

Salt	Preparation	Year	Month	No. of birds	Av. cure days	γ/day dose	Colour reaction	Remarks
HCl	12. 7	1933	—	8	5·0	2·0	5·8	($HgSO_4$ technique)
	17. 0	1933	—	7	6·0	1·67	5·0	—
	92 A 50	1933	Sept.–Oct.	9	5·0	2·0	5·0	—
		1934	Mar.–Apr.	12	3·8	2·63	—	—
		1934	July	13	5·4	1·85	—	—
	19 A	1934	Feb.	6	4·7	2·13	6·0	—
		1934	Feb.	8	4·8	2·08	4·0	High M.P. SO_4 to HCl
	18	1934	Feb.	5	6·2	1·61	6·0	Low M.P. SO_4 to HCl.
	93	1933	Sept.–Nov.	10	4·3	2·33	—	—
	30. 10	1934	May	20	6·3	1·59	5·0	—
	34 A 3	1934	July	15	4·9	2·04	5·8	—
				113	5·093	1·96		

For the whole 113 tests, the mean duration of cure will only exceed 5·093 ±0·250 once in 100 times. The average value for the day dose of free base is 1·54γ.

HCl	V. Veen (1)	1933	Sept.	11	4·0	2·5	6·2	—
	V. Veen (2)	1934	Jan.–Feb.	15	5·3	1·89	6·0	—
	Windaus (2)	1934	June	12	6·1	1·64	4·9	—
HNO_3	7	1933	Nov.	9	5·1	1·96	4·0	—
H_2SO_4	19 A	1934	Feb.–Mar.	11	5·2	1·92	3·0	High M.P.
	19	1934	Feb.	9	6·3	1·59	5·5	Low M.P.
	19	1934	Feb.–Mar.	9	4·0	2·5	4·5	Low M.P.
	24α	1934	Mar.–Apr.	10	4·8	2·08	4·0	—
	24β	1934	Mar.–Apr.	8	5·0	2·0	4·0	—

Examples of tests.

30. 10. May, 1934. Days cure. 10, 9·5, 13, 5, 10, 4, 3, 10, 7, 2, 3, 7, 3, 11, 3, 3, 4, 3, 3, 12.
92 A 48. Mar.–Apr. Days cure. 4, 4, 2, 3, 3, 2, 6, 8, 3, 4, 2, 4.

The sulphate from the average figures appears more active, but the difference is not statistically significant. It is clear that by this test the preparations Van Veen (1) and (2) and Windaus (2) are identical within the limits of error.

Table IV.

			Birds	Days	Day cure
30. 10	June, 1934	Per os	14	4·35	2·3 [*s*]
	May	Injection	—	6·1	1·64
Windaus	June	Per os	9	4·3	2·32 [*s*]
	June	Injection	12	6·1	1·64

[*s*] simultaneous test.

Administration by mouth and by injection have also been compared in simultaneous tests. With extracts of the International Standard, results by the two routes are practically identical (see a paper in course of preparation).

In each case the result by injection is higher; this has been found previously. No stress can be laid upon it owing to the variation.

The average estimate of activity is $1{\cdot}96\gamma$ per day dose for the hydrochloride, and $1{\cdot}17^1 \times 1{\cdot}96\gamma$ per international unit $= 2{\cdot}29\gamma$ or 437 units/mg. The limits outside which the average for such a series of tests could be expected to fall less than 1 in 100 times will be 490–380 units/mg.

These values lie sufficiently close to those of others (estimated at 500 units/mg.) to give confidence that substantially the same activity has been reached.

The above tests have all been made with doses of the same amount. The values obtained agree well with the colorimetric tests and also fairly well with the catatorulin tests and ultraviolet absorption methods. There seems little doubt that they are substantially reliable.

Some discrepancies. Nevertheless, the curative pigeon test is not satisfactory with injection of crystalline vitamin. In previous work from this laboratory, with less pure vitamin given by mouth, it has been found that curative tests by the day dose method gave reliable answers. This was supported by Burn *et al.* [1933] in a comparison of their testing methods with our own. Further the day dose technique is also substantially supported by Birch and Harris [1934]. On the other hand, Williams and Eddy [1933] have criticised our methods in relation to the tests upon preparation Windaus 1 in view of the difference in dose used. This particular criticism was not well founded because with the smaller dose of 10γ in the case of Windaus 1 (by mouth) proper cures could not be effected; there was other evidence too of a lower activity in this sample. The criticism that wide variations occur by the day dose method for injected vitamin receives support from some recent comparisons of our own.

Table V. *Injections of vitamin B_1; simultaneous tests in each case.*

Preparation	Time	Amount	No. of birds	Average days of cure
52 A 15	May, 11–14	5	4	4·2
		10	4	3·7
30. 10	June, 14–16	5	24	5·2
		10	19	6·3
Windaus 2	June, 2–6	5	11	3·3
		10	12	6·1

Upon these figures for 5γ injections, the activity for hydrochloride 30. 10 might be considered to be of the order of 1γ. Small doses injected are evidently liable to give inaccurate results.

We have evidence here that the curative test does not work for injected crystalline vitamin. The matter justifies caution and requires more investigation. In our present opinion, the most reliable biological test for crystalline vitamin will ultimately prove to be the catatorulin test upon avitaminous brain, in which the individual variation is eliminated.

Method of testing and errors.

The probable value of the mean has been calculated for all the hydrochloride results pooled and is given in Table III together with the standard deviation of the figures considered as a whole. This value is considerably higher than that

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[1] 1·17 day dose = 1 international unit upon average.

calculated for groups such as the 20 animals of 30. 10. We have refrained from discussing the individual averages further, because we are doubtful whether the standard formulae for a normal distribution apply to this case. At Mr R. B. Fisher's suggestion, we have found that the results when plotted form a skew-shaped distribution curve, and we wish to analyse this further.

It will be noticed that some comparisons of activity have been made by the method of alternate dosing in agreement with the latest modern practice [Burn *et al.*, 1933]. Though the general value of this method of control is indisputable, in the particular case here considered, there is much to be said for an average of tests made throughout the year. In our practice, successive batches of birds are put down weekly, 40 or 60 in number, birds from which develop symptoms in 18–30 days. Some of these will show temporary cures; by the time that birds are ripe for test we are really presented with a random selection of animals, which have been on the diet for variable periods. Thoroughly shuffled results are equally likely to be reached by alternating batches of control and batches of unknown birds as in the method of strict alternate dosing, provided that such tests are made within a reasonable period of time.

Catatorulin tests.

These were done by the method of Passmore *et al.* [1933] as modified (see Appendix II).

Table VI. *Catatorulin tests.*

Unknown vitamin preparation	Exp.	Standard (s) equivalent	Average
Van Veen 1	505	$0{\cdot}6\gamma = 0{\cdot}65\gamma$	
	510	$0{\cdot}4\gamma = 0{\cdot}45\gamma$	
	513	$0{\cdot}4\gamma = 0{\cdot}5\gamma$	10γ Van Veen $1 = 11{\cdot}5\gamma$ (s)
Van Veen 2	547	$0{\cdot}4\gamma = 0{\cdot}58\gamma$	
Windaus 2	623	$0{\cdot}35\gamma - 0{\cdot}34\gamma$	
	627	$0{\cdot}5\gamma = 0{\cdot}6\gamma$	10γ Windaus $2 = 10{\cdot}8\gamma$ (s)

The standard preparations were 64. 19 for 505, 510, 513; 92 A 50 for 547; 30. 10 for 623, 627. Na pyrophosphate (p_H 7·4) was used in experiments 510, 623, 627. The constants employed for calculation were $R = 20 \times 10^{-2}\gamma$ in all experiments; $k_c = 10 \times 10^{-2}\gamma$ in experiments 505, 513, 547; in experiment 623, $k_c = 30 \times 10^{-2}\gamma$, and in experiment 627 $20 \times 10^{-2}\gamma$; values in experiment 510 were read from an empirical curve.

Oxygen uptakes, μl./g./hr.

	Standard		
Exp.	$0{\cdot}3\gamma$	$1{\cdot}0\gamma$	Unknown
505	955	1090	1045
510	1100	1320	1175
513	700	800	748
547	1120	1268	1205
623	776	1025	810
627	1034	1255	1165

The results leave no doubt that the preparations have substantially the same activity; there is a tendency for the Van Veen material to give a higher value, which is interesting in view of his claim that his preparation is more active. One unreliable test had to be rejected for Van Veen 2.

The effect of crystalline vitamin B_1 on the growth of rats.

The potency of crystalline vitamin B_1 preparations has also been determined by the effect upon the growth of young rats. The rats weighing 30–40 g. were fed on a pure synthetic diet (rice starch 70, caseinogen 20, salt mixture 5, agar-agar 3 and cod-liver oil 3) supplemented with the other factors of the vitamin B complex in the form of autoclaved alkaline marmite. On such a diet, the rats show an initial rise in weight followed by a rapid decline culminating in convulsions and death. When in the convulsive state or on the verge of convulsions, the rats were given the dose of vitamin B_1. The effect of varying doses of the vitamin on the growth of rats is shown in Table VII.

Table VII. *Effect of vitamin B_1 on young rats**.

Vitamin B_1 dose per day	No. of rats	Duration of Exp. (days)	Average growth per week (g.)	σ
1·0	5	18–21	4·64	2·55
1·5	7	17–19	5·24	2·47
2·5	18	17–28	12·7	2·26
5·0	20	14–35	13·94	2·85
6·0	4	10–14	17·5	2·88
10·0	7	21–28	16·34	3·85

* Tests by J. R. O'Brien. σ = Standard deviation.

The results indicate that doses of vitamin B_1 of the order of 1γ suffice to maintain the young rat; after 2–3 weeks on a 1γ dose of vitamin B_1, the rats tend to weaken and to become moribund. Doses of 2·5–5γ are, however, adequate to promote practically normal growth in the young rat. Continued daily dosing of the rats leads to regular growth (Fig. 2). From Table VII, it is evident that a daily dose of 2·5–5γ of crystalline vitamin B_1 is necessary to promote growth up to maximum weight in rats supplied with the other factors of the vitamin B complex under our experimental conditions. It is also clear that doses in excess of this amount have relatively little extra growth-promoting effect.

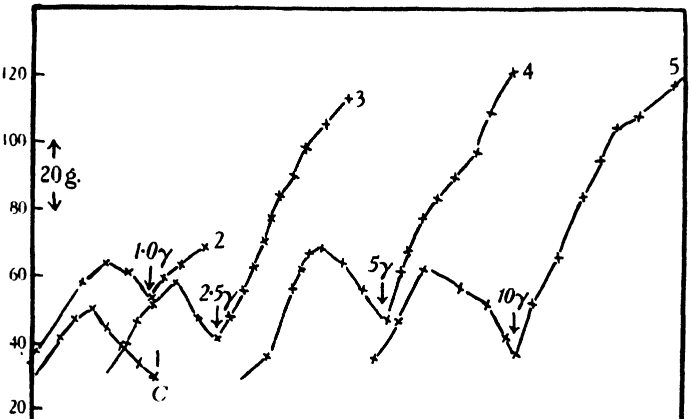

Fig. 2. This shows the effect of varying amounts of crystalline vitamin B_1 hydrochloride upon the growth of young rats of the same sex. Curve 1 shows the rise and decline of weight of the control rat which went into convulsions and died at the point C. Curves 1, 2 and 3 are weight curves for rats of the same litter, 4 and 5 for rats of another litter to which vitamin B_1 was administered at the time marked by the arrow. The control of these two latter behaved like that of 2 and 3.

These figures mean that 1·3 pigeon doses (1·1 international unit) suffices for nearly maximum growth and 2·6 pigeon doses produce improvement after 90 g. weight. These figures are in good agreement with the values of Reader [1930] who usually gave 2–3 pigeon doses to each rat to get normal growth. The lower estimate of 1γ for healthy growth in young rats (by Ohdake [1934]) does not necessarily indicate greater activity of his preparation as compared with ours, because the diet was different and reference to the curves shows that 1γ did not maintain growth well beyond 80–90 g. Ohdake's estimate for pigeons agrees substantially with our own.

Vitamin B_2 concentrates prepared from lead acetate precipitates.

In some cases, with complete success, we have substituted for alkaline autoclaved marmite a vitamin B_2 concentrate prepared from the lead acetate precipitates (a by-product in our large scale process [Kinnersley *et al.*, 1933]) by hydrolysis with acid. The vitamin B_2 complex was shown to be stable to acid by Chick and Roscoe [1929] and Narayanan and Drummond [1930].

Preparation. 600 g. moist lead acetate precipitate [Kinnersley *et al.*, 1933] are treated with 600 ml. H_2O and 300 ml. 20% sulphuric acid. The whole is boiled in a 1500 ml. beaker for 1½ hours with stirring. It is cooled and filtered from the lead sulphate. 20 % NaOH is then added to p_H 3·0 and the mixture is treated with H_2S and filtered. It is usually stored in the dark in cold store until required; any sodium sulphate crystallising out is removed. Before using, H_2S is removed by boiling. The usual volume is 900 ml. for 600 g. precipitate (≡10 kg. original yeast).

1 ml. of this vitamin B concentrate gives maximum effects, 0·5 ml. suffices as a source of all B_2-factors. This concentrate has been both mixed with the diet and given by mouth with identical results. Together with crystalline vitamin B_1, it has cured 23 rats of the dermatitis symptoms of vitamin B_2 deficiency. This concentrate is now being used in attempts to elucidate the problem of factor B_{4a}.

The problem of vitamin B_4.

The very definite results obtained by addition of pure crystalline vitamin B_1 alone raise acutely the question of the meaning of the previous work upon vitamin B_4. The following alternative explanations are possible: (1) Vitamin B_1 in excess can act as a substitute for vitamin B_4. (2) Vitamin B_1 must be present either in the diet or in the rat, *i.e.* by storage. So far as can be ascertained, no change has been made in the experimental diet except for possible variations in the marmite; to the breeding rats, smaller amounts of liver have been given, otherwise the diet has been as before, mixed corn, milk and lettuce twice weekly. Of course, there is no guarantee that climatic conditions have not influenced the composition of the corn *etc.* in some unknown way. (3) The explanation of the previous experiments [Reader, 1929; 1930] must have been in error. If we examine carefully the evidence for vitamin B_4 presented by Reader, we find in reality that two vitamin B_4 factors have been proposed. B_{4a}, the original, was a factor necessary for the growth of young rats; like the flavins, it was precipitated by mercuric sulphate. Believing that no change had been made in the factor studied, a reasonable view at the time, Reader [1930] changed the test and finally worked with a curative test for adult rats. The factor B_{4b} so studied was found in the same crude acid charcoal concentrates as B_{4a}. There is however no real guarantee that the factors B_{4a} and B_{4b} were identical. Considering the factor B_{4a}, the important point of the original work was that some factor other than vitamins B_1 and B_2 (as then understood) was essential for the rat. This point is abundantly supported by recent work; many investigators have recognised a need for a B_4-factor among whom may be quoted Moore *et al.* [1932], Halliday [1934] (rat), Keenan *et al.* [1933] (young chickens).

It is now generally believed that flavin is part of the original vitamin B_2 [György *et al.* 1934; György, 1934; Chick and Copping, 1934]; but it is not excluded that the factor B_{4a} might have been some manifestation of flavin and factor Y either separately or together; especially as these are found in crude vitamin B_4 concentrates[1]. The explanation of the factor B_{4b} is more obscure. Vitamin B_1 concentrates were originally obtained which failed both to cure the special symptoms and to increase the weight of rats. Such concentrates cannot now be prepared. The ataxic symptoms described by Reader in the adult rat (not the young rat) have been reproduced and cured by crystalline vitamin B_1 [O'Brien, 1934]. But the condition of pink feet has been of late uniformly absent. This may be a deciding factor; until it can be reproduced, a considered judgment upon the problem of factor B_{4b} must be suspended. It may be noted that Halliday described ataxic symptoms in adult rats upon milk diets which were attributed to vitamin B_4 deficiency. The pink paws are suggestive of some features of the egg-white syndrome [Boas-Fixsen, 1931]. We cannot exclude the possibility that the symptoms had a toxic origin and that factor B_{4b} may be an antitoxic agent; though we are loth to complicate the problem with such a view at the present stage.

SUMMARY.

The information in this and the accompanying paper by Holiday [1935] can be summarised in tabular form (Table VIII).

Table VIII.

Method of test	Our HCl	Windaus II	Van Veen I	Van Veen II
Pigeon (day dose)	1·96γ	1·64γ	2·5γ	1·9γ
Catatorulin test	10γ	9·9γ	11·5γ	15γ
ϵ/c spectroscopic measurement (Holiday)	45	45·6	39·3	40·4
Colour test	10γ	9·6γ	12·4γ	12γ

1. Vitamin B_1 hydrochlorides from different sources are shown to be identical by various methods of test. An average activity of 380–490 international units/g., average 437, has been found. Analyses agree best with the formula $C_{12}H_{16}ON_4S_3.2HCl$, but they are not quite consistent with this.
2. One nitrate and two sulphates of vitamin B_1 have been prepared with distinct and sharp melting-points.

We are indebted to the Medical Research Council for personal grants and for grants for expenses, and to M. Kempson and R. W. Wakelin for skilful assistance.

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[1] György's vitamin B_6 is here considered to be identical with factor Y.

APPENDIX I.

Table IX. *Analyses of vitamin B_1 hydrochloride by H. W. Kinnersley and J. R. O'Brien.*

Date	Preparation	Amount mg.	% C	% H	% N	% S	% Cl
24. v. 33	57. 7	2·218	42·33	5·88	—	—	—
20. v. 33	57. 7	1·805	—	—	14·6	—	—
25. v. 33	57. 7	1·647	—	—	—	—	21·0
18. i. 34	68 A 11	2·108	—	—	15·56	—	—
			—	—	15·13	—	—
19. x. 33	68 A 11	7·21		—	—	9·82	—
	69. 32	22·72	—	—	—	—	20·7
24. v. 33	78. 33	1·718	42·06	7·02	—	—	—
20. v. 33	80. 37	1·817	—	—	15·15	—	—
23. v. 33	80. 37	2·214	—	—	—	9·03	—
24. v. 33	83. 7	2·067	41·6	5·97	—	—	—
21. ix. 33	70 A 13	1·792	—	—	—	9·80	—
	93 A 47	13·2	—	—	—	—	20·66
21. ix. 33	93 A 47	2·670	—	—	—	9·77	—
4. x. 33	93 A 47	5·166	—	—	—	10·00	—
9. x. 33	93 A 47	6·09	—	—	—	10·12	—
14. x. 33	93 A 47	6·64	—	—	—	9·72	—
20. x. 33	92 A 50	5·60	—	—	—	9·72	—
10. x. 33	74. 26	4·25	—	—	—	—	21·14
2. v. 34	70 A 6	6·53	—	—	—	—	21·2
27. i. 34	68 A 11	2·550	42·94	5·6	—	—	—

Analyses of vitamin B_1 hydrochloride obtained by conversion of the sulphates.

Date	Preparation	Amount mg.	% C	% H	% N	% S	% Cl
From the sulphate of low M.P.:							
13. ii. 34	19 A 51	1·998	—	—	15·92	—	—
14. ii. 34	19 A 51	2·815	41·68	5·45	—	—	—
28. iii. 34	18. 51	1·653	—	—	15·92	—	—
		1·897	42·27	8·0	—	—	—
From the sulphate of high M.P.:							
28. iii. 34	18. 37	3·415	41·9	5·53	—	—	—
		1·851	—	—	17·32	—	—
27. iv. 34	18 A 3	2·250	—	—	15·37	—	—
		3·43	41·34	8·4	—	—	—
					Average values		
Kinnersley and O'Brien ...			41·9	6·6	15·6	9·75	20·94
Schoeller			42·2	5·7	15·1	9·5	20·92

All values are corrected for water content and residue of vitamin. Sulphur values were determined by modified Pregl sulphur analysis [Kinnersley and O'Brien, 1934].

APPENDIX II.

Notes upon the catatorulin test for vitamin B_1 by R. A. Peters.

In this test, the power of vitamin B_1 to restore a specific *in vitro* respiration in avitaminous brain tissue is used. Experience with the method of Passmore *et al.* [1933] has led to the introduction of changes. Working with lactate only, the differences between $0{\cdot}3\gamma$ and 1γ vitamin B_1 are often obscured by the variation in duplicate samples. It is better to take advantage of the magnification in the catatorulin effect induced (*a*) by the use of pyrophosphate [Peters and Sinclair, 1933] and (*b*) by warm mincing [Peters *et al.*, 1935]. The latter requires considerable modification of the empirical equation, previously suggested. Details of procedure must be controlled at each point, otherwise unsatisfactory duplicates are obtained.

Procedure. Pigeons are cured at least once for periods over two days with a known or experimental sample of vitamin B_1. They are rendered more free from vitamin B_1 in this way. When symptoms reappear, the head is removed by guillotine, excess blood allowed to drain from the head upon filter-paper for 2–3 mins. The brain is then exposed by cautious excision of the top of the skull. The excised brain is placed upon a glass water-jacketed funnel (37° ± 2°) and the required parts, usually cerebrum, optic lobes and lower parts, minced with a bone spatula for 2–3 mins. and thoroughly mixed. Quantities of about 100 mg. are selected and placed successively in each tared bottle containing the Ringer-lactate-pyrophosphate solution. It is so arranged that one half of the bottles are filled before the other half. In this way, differences occurring as the tissue stands are minimised. The tissue is allowed to remain without disturbance in a small ball; the bottles are then re-weighed. When the weighing is complete, the tissue is divided into small fragments with a glass rod or crusher; care is taken to remove minimum amounts of fluid upon withdrawing the crusher. The divided tissue is allowed to stand for 1–2 mins. and addition of vitamin B_1 is then made. The vitamin B_1 is prepared by evaporation to dryness of an acid alcoholic solution in a small tube *in vacuo*; the dry vitamin is dissolved in the Ringer-phosphate-lactate-pyrophosphate[1] medium.

Bottles are then twice evacuated to 70 mm. and filled with oxygen; duplicates are evacuated in different batches again to control changes occurring upon standing. As each batch is filled with oxygen the taps are turned and the apparatus briefly shaken to saturate the Ringer with oxygen. The apparatus are all together quickly placed in the bath and shaken for 12 mins. before taking the first reading.

Readings can be taken at half-hour periods as before. In some cases, the first period has been 15 mins. and the subsequent readings for half-hourly periods.

Exp. 761 shows an experiment with pyrophosphate, the values being the mean of duplicates. The figures represent O_2 uptakes (μl.).

	Period hrs.				
Period ...	1	2	3	4	5
Vitamin	0–¼	¼–¾	¾–1¼	1¼–1¾	1¾–2¼
Nil	2060	1650	1360	1045	850
0·5γ	2350	2175	1900	1645	1460
1·0γ	2400	2300	2040	1860	1645
2·0γ	2410	2250	2100	1870	1695

Within the first hour, ¼–1¼, the differences are of the same order as formerly. We can calculate *V* from the Michaelis equation originally given by Passmore *et al.* [1933], and, as in this case, we often get fair agreement.

	v	*V*
For 0·0γ	1505	2260
0·5γ	2037	2330
1·0γ	2170	2350

During the second hour, pyrophosphate gives a largely increased vitamin effect, *i.e.* 66 % over the control for 1γ vitamin as compared with 44 % in the first hour. This increase varies to some extent with the condition of the tissue. The reactions are complicated and do not lend themselves at present to simple conceptions (*cf.* Peters *et al.* [1935] for a partial analysis of this). I believe it best to use a frankly empirical equation to fit individual cases. In Exp. 761 and some others, the points: 0·0, 0·5γ and 1·0γ are fitted approximately by the Michaelis equation if $kc=30$ and $R=20$.

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[1] In some cases, Na pyruvate, 1 mg./3 ml. has been added, but it is not certain that this is an mprovement.

For the second hour, $kc=30$, $R=20$.

	v	V
0·0γ	950	2370
0·5γ	1550	2220
1·0γ	1750	2190

1γ as in the original work evidently gives approximately maximum effects so that the curve is discontinuous after 1γ, *i.e.* $2\gamma \equiv 1\gamma$.

In the cases which have been investigated, the approximate answer is not usually in any doubt. With pyrophosphate and warm mincing a quite satisfactory comparison of activity can often be obtained with the mean of duplicate values, owing to the much larger relative change in the oxygen uptake for different vitamin concentrations. I regard this as a most promising method of comparing the strengths of vitamin solutions, though more work is required to determine the most suitable equation for the new conditions.

REFERENCES.

Barnes and Peters (1932). *Biochem. J.* **26**, 2203.
Birch and Harris (1934). *Biochem. J.* **28**, 602.
Bernal and Crowfoot (1933). *Nature*, **131**, 911.
—— —— (1934). *Nature*, **134**, 809.
Boas-Fixsen (1931). *Biochem. J.* **25**, 597.
Burn, Coward, Ling and Morgan (1933). *Biochem. J.* **27**, 1719.
Chick and Copping (1934). *Chem. and Ind.* **53**, 874.
—— and Roscoe (1929). *Biochem. J.* **23**, 506.
György (1934). *Nature*, **133**, 498.
—— Klaveren, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 236.
Halliday (1934). *J. Biol. Chem.* **106**, 29.
Heyroth and Loufbarrow (1934). *Nature*, **134**, 461.
Holiday (1935). *Biochem. J.* **29**, 718.
—— Kinnersley, O'Brien and Peters (1934). *Chem. and Ind.* **53**, 1024.
Jansen and Donath (1926). *Proc. K. Acad. Wetensch. Amsterdam*, **29**, 1390.
—— Kinnersley, Peters and Reader (1930). *Biochem. J.* **24**, 1824.
—— Wibaut, Hubo, and Wierli (1933). *Rec. Trav. Chim. Pays-Bas*, **52**, 366.
Keenan, Kline, Elvehjem and Hart (1933). *J. Biol. Chem.* **103**, 671.
Kinnersley and O'Brien (1934). *Chem. and Ind.* **53**, 830.
—— —— and Peters (1932). *J. Physiol.* **75**, 17 *P.*
—— —— —— (1933). *Biochem. J.* **27**, 232.
—— —— —— (1934). *Chem. and Ind.* **53**, 452.
—— and Peters (1928). *Biochem. J.* **22**, 419.
—— —— (1929). *Biochem. J.* **23**, 1126.
—— —— (1930). *Biochem. J.* **24**, 711.
—— —— (1934). *Biochem. J.* **28**, 667.
Malcolm (1933). *Quart. J. Exp. Physiol.* **23**, 83.
Moore, Plymate, Andrew and White (1932). *Amer. J. Physiol.* **102**, 566.
Narayanan and Drummond (1930). *Biochem. J.* **24**, 24.
O'Brien (1934). *Chem. and Ind.* **53**, 452.
Ohdake (1932). *Proc. Imp. Acad. Japan*, **8**, 179.
—— (1934). *Bull. Agric. Chem. Soc. Japan*, **10**, 71.

Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Peters and Philpot (1933). *Proc. Roy. Soc. Lond.* B **113**, 48.
—— and Sinclair (1933). *Biochem. J.* **27**, 1910.
—— Rydin and Thompson (1935). *Biochem. J.* **29**, 53.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930). *Biochem. J.* **24**, 77, 1827.
Seidell (1929). *J. Biol. Chem.* **82**, 633.
—— and Smith (1933). *J. Amer. Chem. Soc.* **55**, 3380.
Van Veen (1932). *Rec. Trav. Chem. Pays-Bas*, **51**, 265, 269.
—— (1933). *Z. Physiol. Chem.* **208**, 125.
—— (1934). *Natur*, **133**, 137.
Williams and Eddy (1933). *Carnegie Inst. Year Book*, 320.
—— Waterman and Keresztesy (1934). *J. Amer. Chem. Soc.* **56**, 1187.
Windaus, Tschesche and Ruhkopf (1932). *Nachrich. Göttingen*, 342.
—— —— —— Laquer and Schultz (1933). *Z. physiol. Chem.* **204**, 123.

LXXXII. IMPROVED YIELDS OF VITAMIN B_1.

BY HENRY WULFF KINNERSLEY, JOHN RICHARD O'BRIEN AND RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(*Received February 1st, 1935.*)

THE yields previously reported [Kinnersley *et al.*, 1933] have now been improved and some changes introduced. By using a special yeast supplied by the Distillers Company Ltd., about four times the amount of crystals are obtained from a given amount of yeast; 60 mg. pure hydrochloride may be expected from 50 kg. moist yeast (10 kg. as dry). It is an improvement to adsorb on charcoal at p_H 6·5 as the vitamin seems less liable to damage at this p_H value. The special yeast does not give a clear filtrate on centrifuging after the initial boiling. This can be clarified by the use of more lead acetate, at least double the amount previously recommended. It is apt to filter rather slowly at the baryta stage. The phosphotungstate stage works with precision; we notice that others have confirmed its value [Williams *et al.*, 1934]; the formaldehyde-azo-test is here especially useful and we place increasing reliance upon it [Kinnersley and Peters, 1934].

In the final stages of crystallisation, after removing the bulk of the crystals with acid alcohol (HCl, p_H 3), containing about 10 mg. of crystals in each ml., the remaining vitamin may be obtained as crystals by concentrating the filtrates to about half the volume and adding light petroleum (B.P. 40–60°) to the hot solution to faint cloudiness and cooling. Some further 15 % yield may thus be obtained.

As previously recorded (and as confirmed by Williams *et al.* [1934]), loss occurs at the gold stage, presumably owing to oxidation. We have tried to eliminate this by addition of cyanide but have obtained no certain improvement; in some experiments cyanide decreased the yield. Exp. 1 is an example of several experiments of this type.

Exp. 1. *The precipitation of the activity by gold chloride in presence of KCN.* The alcohol was removed *in vacuo* from a volume of the 99 % alcohol solution equivalent to ½ cwt. yeast (20,000 doses); 15 ml. of water were added and about 3 ml. of 10 % gold chloride and 1 ml. 10 % KCN alternately and in small and proportionate volumes at a time; the 30 ml. to be centrifuged were meanwhile cooled in ice. After a few minutes the precipitate was centrifuged off and without being washed was suspended in 15 ml. $N/200$ HCl, and H_2S was passed through for a period of 1 to 2 hours. The sulphide precipitate was filtered under micro-conditions and the filtrate concentrated in a small evaporating basin on the water-bath with 1 drop of 10 % $BaCl_2$ to about 0·5 ml. or less. During the last stages, the side of the basin was washed with alcohol, with which solvent the residue was finally removed into a small 5 ml. tared tube. In this tube most of the water was removed by heating and shaking in the bath—any inactive precipitates were centrifuged off and the solution was allowed to crystallise in the cold chamber for 24 hours or longer.

Equal amounts representing 25 kg. yeast were worked up with and without KCN. The yields were, with KCN: 21·3, 25·6, 30·5; without KCN: 35 mg.

The gold stage seems indispensable for crystallisation; loss is best avoided by minimum times of precipitation (5 mins.) and the use of cooling. We have been unable to convince ourselves that the passage of nitrogen is definitely helpful.

Table I gives the present yields. It must be realised that there are also proportionately increased yields of cruder vitamin B_1 present in the 50 % alcoholic extracts of charcoal which we find very useful in other work.

Table I. *Vitamin B_1 yields from special baker's yeast (moist).*

The amounts of vitamin hydrochloride present unless otherwise stated are judged by colour test in which $2\gamma = 1$ dose vitamin.

			Pigeon doses			
Date	Batch	Amount kg.	99 % alcohol stage	Phosphotungstate stage (p_H 5)	Pure crystals mg.	mg./50 kg.
6. ii. 33	87	25	20,000	30,000	34·6	73·2
6. ii. 33	89	200	150,000	150,000 145,000 (Bird)	222·0	55·5
7. ii. 33	89	100	80,000 90,000 (Bird)	—	130	65
4. iii. 34	33	100	100,000	90,000	131·5	65·7
8. iii. 34	47	200	220,000 230,000 (Bird)	140,000	210	52
					Average	62·3

SUMMARY.

Improvements in the yield of pure crystalline vitamin B_1 hydrochloride are described.

We wish to thank the Medical Research Council for personal grants and for a grant for expenses. We are also indebted to the Distillers Company Ltd. for the facilities provided. We also thank T. Clarke, T. T. Cox, M. Kempson, W. T. Turner and R. W. Wakelin for skilful assistance.

REFERENCES.

Kinnersley, O'Brien and Peters (1933). *Biochem. J.* **27**, 232.
—— and Peters (1934). *Biochem. J.* **28**, 667.
Williams, Waterman, Keresztesy (1934). *J. Biol. Chem.* **56**, 1187.

Note. In the paper by Kinnersley and Peters [1934], on page 668, line 28, *delete* "of p_H 5".

LXXXIII. NOTE ON THE ULTRAVIOLET ABSORPTION OF CRYSTALLINE PREPARATIONS OF VITAMIN B_1.

By ENSOR ROSLYN HOLIDAY.

From the Medical Unit and Physiological Laboratories, the London Hospital and the Department of Biochemistry, Oxford.

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Since Peters and Philpot [1933] stated that two samples of crystalline vitamin B_1 prepared at Oxford showed absorption maxima at 247 $m\mu$, whereas other workers [Heyroth and Loufbarrow, 1931] had correlated vitamin B_1 activity with absorption about 260 $m\mu$ it seemed doubtful whether any relation existed between absorption in the ultraviolet region of the spectrum of vitamin B_1 preparations and their biological activity.

Subsequently the opportunity occurred of examining several crystalline samples of vitamin B_1 prepared independently in three different laboratories, namely two samples from Prof. Peters, two from Prof. Windaus, and two from Dr Van Veen to whom grateful acknowledgment is made for the opportunity of making the comparison.

The object of the investigation was to discover what differences, if any, in the ultraviolet absorption existed between the samples of vitamin B_1 at hand when examined under similar conditions. The observation of Peters and Philpot [1933] that on rendering an acid solution of vitamin B_1 alkaline there was a shift of the maximum absorption to shorter wave-lengths and the appearance of a new and labile maximum (330–340 $m\mu$) suggested a basis of comparison. Since comparison of the changes of absorption in samples of unknown constitution obtained by varying p_H is likely to be more sensitive to differences in composition than a single determination at a given p_H, absorption measurements were made on all six samples in $N/200$ HCl at successive time intervals after bringing to a p_H between 11·0 and 12·0, and then subsequently after reacidification.

The effect on the absorption of varying the solvent was also tried.

Experimental.

The absorption measurements were made with a Hilger quartz E 316 spectrograph with Spekker photometer [Twyman, 1931; Twyman and Allsopp, 1934], using a condensed spark between tungsten steel electrodes as light source.

A weighed amount of each sample was originally dissolved in 75 % alcohol containing $N/200$ HCl and examined in a suitable layer thickness (0·5–2·0 cm.). The solution was diluted with 75 % acid alcohol to give a suitable density value in a layer of 5 cm. thickness and measured again. It was found that over this small range of dilution (22 to 10 times) Beer's law is followed within the limits of determination (± 2 %).

The 5 cm. cell was of standard Hilger type with centre filling funnel. For the determination of the alkali change a suitable volume (obtained by trial) of *N* NaOH was added through the funnel and the tube shaken for a few seconds, the thumb occluding the funnel opening, the enclosed bubble acting as mixer. The absorption was measured at once and then at intervals of 5 mins.

The time taken to expose a plate with the method here used is about 3 mins., and from the results of succeeding measurements it was evident that during this time some change in absorption had taken place. The absorption curves of the alkaline solutions are therefore mixed curves and cannot be taken as having accurate quantitative significance. Nevertheless, comparison of the curves with those obtained by Peters and Philpot [1933], who used a method requiring only 5–15 secs. exposure [Philpot and Schuster, 1933], shows quite good agreement.

RESULTS.

The results of the determinations are grouped in Table I, which shows the values obtained for the six samples in *N*/200 HCl in 75 % alcohol. The extinction coefficient has its usual meaning and *c* is in g. per litre. In order that the extinction coefficients of the sulphates and hydrochlorides should be comparable, they have been reduced to correspond to concentration of free base. The close correspondence between 61 A (15) and 64 (19) and Windaus II is very remarkable, and after reducing to free base value this is extended to 26 (59). The discrepancy in 26 (52) is discussed by Kinnersley *et al.* [1935]. The samples Van Veen I and II and Windaus I are also very similar and differ from the former group in having a markedly lower extinction coefficient and a maximum at λ 245 instead of λ 247 *mμ*.

Table I.

Source and designation	Salt	ϵ/c for salt	% free base in salt	ϵ/c for free base	Wave-length of maximum, *mμ*
1. 26 (59)	Sulphate	40·2	65	61·9	247
2. 26 (52)	,,	31·8	60	53	246
3. 61 A (15)	Hydrochloride	46·4*	78·5	59·1	247
4. 64 (19)	,,	45·0*	—	—	247
5. Windaus I	,,	38·8	—	—	245
6. Windaus II	,,	45·6 45·3†	78·5	60·6	247
7. Van Veen I	,,	39·3	—	—	245
8. Van Veen II	,,	40·4	—	—	245

1–4 Prepared by Kinnersley *et al.* [1935].
* Quoted from Peters and Philpot [1933].
† Determination by Prof. Pohl, private communication by Prof. Windaus.

Fig. 1 shows the absorption curves of 26 (59), Windaus II and Van Veen II in *N*/200 HCl in 75 % alcohol.

All six examples showed the alkali change of Peters and Philpot. As stated above, the method employed is really too slow for the making of measurements of rates of change occupying only about 15 mins. It is not surprising therefore that no difference in this respect was noticeable among the six samples.

The solvent was found to have great influence upon the absorption. Fig. 2 shows different absorption curves obtained with the same sample of vitamin. If the vitamin is dissolved in neutral 80 % alcohol and then acidified, it does not show the same absorption as when dissolved originally in acid alcohol. The same applies to aqueous solutions.

It will be noted that in neutral alcoholic solution the vitamin shows two well-defined bands at 234 and 268 $m\mu$. These correspond very closely with the bands found by Heyroth and Loufbarrow [1934] in some specimens of vitamin B_1 [see also Smakula, 1935]. In simple distilled water the absorption curves obtained with the vitamin are poorly reproducible. The reason for this was traced to the fact that between p_H 5·5 and 7·0 the vitamin is extremely sensitive to change in hydrion concentration, and the slight variations in the p_H of the distilled water used were sufficient to cause marked changes in the absorption curve. All curves obtained under these conditions are mixtures of curves 2 and 3, Fig. 2. The importance of measuring the absorption of the vitamin under standard conditions of hydrion concentration is therefore obvious.

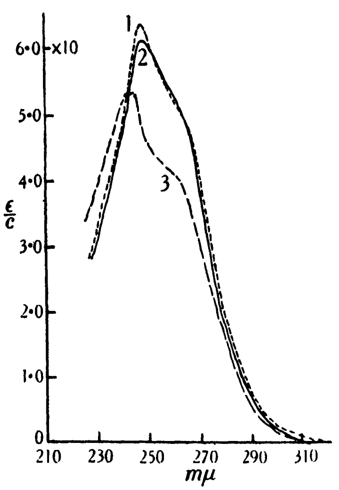

Fig. 1.

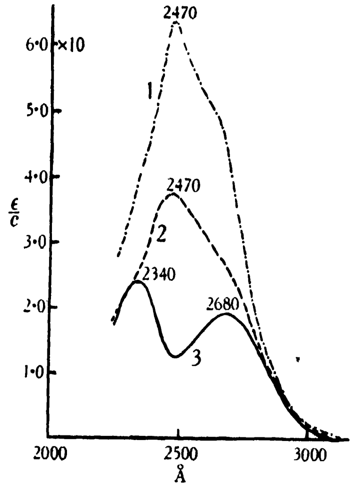

Fig. 2.

Fig. 1. Vitamin B_1. 1 - - - - - 26 (59); 2 ——— Windaus; 3 — — — Van Veen.

Fig. 2. Vitamin B_1 hydrochloride. 1 —•—•— Dissolved originally in acid alcohol; 2 - - - - 80 % alcohol acidified; 3 ——— 80 % alcohol.

Discussion.

It appears that the purest samples of crystalline vitamin B_1 in acid alcohol all show selective absorption with a maximum at 245–247 $m\mu$. That others should have correlated vitamin B_1 activity with absorption at 260 $m\mu$ is difficult to explain. In this regard Prof. Windaus is now in agreement that his specimens show a maximum at 247 $m\mu$ (private communication to Prof. Peters) as against a previously published maximum at 260 $m\mu$.

The marked influence of hydrion concentration on the absorption of vitamin B_1 has no doubt been a source of confusion. There is the additional possibility that in less pure samples the impurities not only may have maxima about 260 $m\mu$, but may also alter the absorption of the vitamin.

It also appears that vitamin B_1 preparations of highest activity give very similar absorption curves. The extinction coefficients, the behaviour of the absorption on changing the hydrion concentration and the maxima of absorption are almost identical.

It is therefore probable that the absorption measured is that of the vitamin B_1 molecule, and that in the three laboratories from which the above samples are derived one and the same entity is being used.

REFERENCES.

Heyroth and Loufbarrow (1931). *Bull. Basic Sci. Res.* **3**, 237.
—— —— (1934). *Nature*, **134**, 461.
Kinnersley, O'Brien and Peters (1935). *Biochem. J.* **29**, 701.
Peters and Philpot (1933). *Proc. Roy. Soc. Lond.* B **113**, 48.
Philpot and Schuster (1933). *Med. Res. Coun. Spec. Rep. Ser.* No. 177.
Smakula (1935). *Z. physiol. Chem.* **230**, 231.
Twyman (1931). *Trans. Opt. Soc.* **33**, 1.
—— and Allsopp (1934). The practice of absorption spectrophotometry. (London.)

LXXXIV. THE WATER-SOLUBLE B-VITAMINS.

IV. THE COMPONENTS OF VITAMIN B_2.

By HARRIETTE CHICK, ALICE MARY COPPING[1]
AND CONSTANCE ELIZABETH EDGAR.[2]

From the Division of Nutrition, Lister Institute, London.

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Note. Vitamin B_2 may be defined as the heat-stable constituent of the vitamin B complex which must be added to diets containing all other dietary essentials, including the antineuritic vitamin B_1, in order to maintain growth and health in the rat and prevent occurrence of dermatitis ("rat pellagra").

THE recent isolation of lyochromes, water-soluble pigments with characteristic yellow green fluorescence, by Ellinger and Koschara [1933] and by Kuhn *et al.* [1933, 1, 2, 3] and the discovery that these pigments are present in materials possessing the biological attributes of vitamin B_2 have given a new impetus to the study of this vitamin. A series of such pigments, having certain well-defined physical and chemical properties in common, has been isolated in pure form from many substances rich in vitamin B_2, *viz.* whey, egg-white, yeast, liver, kidney [Ellinger and Koschara, 1933; Kuhn *et al.*, 1933, 1, 2, 3; Warburg and Christian, 1932; 1933; Karrer *et al.*, 1934].

At first some of these pigments, when administered even in very small doses, were thought to possess the whole nutritive properties of vitamin B_2. Later, however, as purer specimens were available for testing, it became apparent that neither ovoflavin nor lactoflavin nor hepatoflavin [Kuhn *et al.*, 1933, 2, 3; Kuhn, Rudy and Wagner-Jauregg, 1933; György *et al.*, 1933; Euler *et al.*, 1934], when added with vitamin B_1 to a "-B" diet, could supply the full function of vitamin B_2, but that some further supplement was necessary; in other words, that what had hitherto been regarded as vitamin B_2 contained at least two constituents. In these experiments the vitamin B_1 was supplied as an alcoholic cereal extract [György *et al.*, 1934] or as an adsorbate on acid clay prepared from rice polishings by Jansen and Donath's method [Euler, Karrer and Alder, 1934].

The missing supplement for lactoflavin or ovoflavin was provided by the addition to the diet of a yeast extract, from which the B-vitamins or lyochromes had been adsorbed by treatment with fuller's earth [Kuhn *et al.*, 1933, 2; György *et al.*, 1933; Euler, Karrer and Malmberg, 1934] or when vitamin B_1 was supplied as a concentrate made from baker's yeast by the Peters process [György *et al.*, 1934]. The latter observation at first led György and his co-workers to the conclusion that the heat-labile "vitamin B_4" [Reader, 1929; 1930, 1] was the missing factor. "Vitamin B_4" is stated to accompany vitamin B_1 to the end stage of the Peters process, as ordinarily carried out [Reader, 1930, 2]. The conclusion that "vitamin B_4" was involved has, however, recently been abandoned by György [1934] in favour of the idea that the supplement needed by flavin, which is present in the Peters preparation, is a new heat-stable component of the vitamin B complex. This new vitamin György [1934] has called "vitamin

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[1] Working with a part-time grant from the Medical Research Council.

[2] Francis Maitland Balfour Student (Newnham College, Cambridge).

B_6" and has suggested that it may be identical with the "factor Y" described by Chick and Copping [1930, 2] and Roscoe [1930]. This vitamin B_6, together with the flavin, forms a combination having the full biological action formerly attributed to vitamin B_2.

The above forms a very brief summary of work published in a large number of small papers during the last two years. In most of these only scanty details were given of the animal tests which formed the basis of the conclusions drawn as to the relation of the flavins to vitamin B_2. The subject seemed to us to need fuller investigation and the following experiments were begun about a year ago, in the hope of elucidating the nutritive properties of flavin and its relation to the vitamin B complex, not only for maintaining growth, but possibly also for preventing and curing dermatitis in rats.

The lyochromes investigated by us included a specimen of pure lactoflavin D prepared from whey (analysis: C, 52·75, 52·69; H, 5·70, 5·84; N, 14·43, 14·38 %; M.P. 269–273° (uncorr.)) kindly provided by Prof. Ph. Ellinger, and one of pure hepatoflavin from liver (analysis: C, 55·01, 54·85; H, 5·53, 5·65; N, 14·33, 14·32 %; M.P. 275–280°) placed at our disposal by Dr Sydney Smith. These specimens agreed well, both in analysis and melting-point, with those isolated respectively by Kuhn *et al.* [1933, 3] (lactoflavin) and by Karrer *et al.* [1934] (hepatoflavin). We wish here gratefully to acknowledge these gifts. Without them this work would have been impossible.

METHODS.

Diets. The tests for vitamin B_2 were carried out by the methods usual in this laboratory [Chick and Roscoe, 1928]. Young rats, when weaned, received the usual "-B" diet (caseinogen 100, rice starch 300, cottonseed oil 75, salt mixture (McCollum's No. 185) 25, water 500). The diet was heated in a steamer for 3 hours at 100°, in order to cook the starch grains, so that "refection" might be prevented [see Roscoe, 1927]. Vitamins A, D and B_1 were administered separately to each rat daily by capillary pipette, A and D as 0·08 g. cod-liver oil and B_1 as 0·1 ml. ($\equiv$0·6 g. original yeast, dry weight) of a Peters concentrate prepared from brewer's yeast [Chick and Roscoe, 1929].

We have not found any advantage in the elaborate purification of the caseinogen previously adopted [see also Roscoe, 1933] and for most of the present work a commercial sample of "light white casein" was used. A few comparative tests were made with similar "-B" diets containing purified caseinogen, but again no difference was detected either in the extent to which growth was checked or in the incidence of dermatitis.

In some of the diets the rice starch was replaced by an impure sample of maize sugar (containing 85 % dextrose, 1 % ash and *ca.* 14 % maltose, dextrins and other impurities), such as is used in this country in the manufacture of beer. This change was made in order to see if a maize derivative (by analogy with human pellagra) might prove conducive to regular development of the specific skin disorders which at the time were occurring only rarely in our young rats on diets deficient in vitamin B_2. The use of sucrose in the basal diet in place of starch was found conducive to the development of dermatitis in rats deprived of vitamin B_2 by Hogan and Richardson [1934].

Growth tests. Growth frequently ceased immediately from the time the rats received the experimental basal diet; in other cases there was very slow but steady increase in weight for several weeks. The difference appeared to depend on the reserves of B-vitamins with which the young rats were endowed and it has been noted that rats from our colony have appeared to be less sensitive to lack of vitamin B_2 since a generous allowance of dried yeast has been included in the breeding diet. This change was introduced to produce young rats in a more satisfactory condition for work on vitamins A and D. We have therefore tried the effect, with good results, of limiting the B-vitamins, especially vitamin B_2, in the diet of the mothers of litters intended for vitamin B_2 work. No yeast is given during lactation, the mothers' diet consisting of mixed cereals, milk, raw cabbage and carrot, for the first 2 weeks, and for the third week of the "-B" experimental diet supplemented with vitamin B_1 as Jansen and Donath's acid clay adsorbate from rice polishings. If the weight increase of the litter appeared to be too severely checked, milk was given in addition during the latter period and the weaning postponed for a few days, until the weight of the young rats was 30–40 g. This procedure has been successful in producing rats much more sensitive to vitamin B_2 deficiency, showing more regular cessation of growth and a more regular and quicker development of skin disorders.

When the body weight was stationary the daily doses of flavin were administered, with and without the various supplements to be tested, and growth was observed for 3 or 4 weeks or longer.

Table I. *Incidence of dermatitis in young rats receiving a diet deprived of vitamin B_2 from the time of weaning, the mothers being also deprived of this vitamin during the last week of lactation.*

Vitamin B_1 administered separately to each rat as 0·1 ml. (≡0·5 g. yeast dry weight) of a "Peters concentrate" prepared from brewer's yeast.

(*a*) signifies the florid type of dermatitis ("rat pellagra"). ± signifies slight or incipient.
(*b*) ,, the more generalised type. + ,, definite.
0 ,, no symptoms. + + ,, severe.

Result after 9 weeks on the experimental diet.

Litter	Carbohydrate in basal diet	Rat No.	Body weight g.	Incidence of dermatitis Type (*a*)	Type (*b*)	General condition
23	Maize sugar	211	42	+ +	+ +	Very bad, rat died
	,,	214	56	±	0	Good
	,,	215	59	±	+ +	Fair
	,,	218	55	±	0	Good
	Rice starch	212	48	0	±	Fair
	,,	213	41	0	+ +	Very poor
	,,	216	48	+	+ +	Poor
	,,	217	41	0	±	Good
24	Maize sugar	219	72	±	0	Good
	,,	223	67	0	+	Good
	,,	224	57	0	±	Fair
	,,	226	83	0	+	Good
	Rice starch	220	59	0	+ +	Poor
	,,	221	61	0	+ +	Poor
	,,	222	53	±	±	Good
	,,	225	45	+ +	+	Poor

Cure of dermatitis. The irregularity in the occurrence of dermatitis in rats deprived of vitamin B_2, for which no adequate explanation has been forthcoming, has hitherto been a hindrance to the study of this syndrome [see Roscoe, 1933; Hogan and Richardson, 1932]. We have now concluded that differences in the reserves of vitamin B_2 possessed by the young animals at the beginning of the experiment are the principal cause of this uncertainty. The substitution of impure maize sugar for starch in the basal diet has also seemed to encourage the regular incidence of dermatitis.

The skin disorders which have been observed by us in rats suffering from deficiency of vitamin B_2 (using this term in the sense defined at the beginning of this paper) have shown the following two types [Chick and Roscoe, 1928, p. 795; Chick and Copping, 1930, 1, p. 933; see also György, 1934]:

(*a*) A florid dermatitis, roughly symmetrical, in which there is redness, swelling and oedema of the skin of the extremities (ears, digits of the fore and hind paws) and sometimes also of the nose and corners of the mouth; this may spread later over the limbs and trunk with cracking and desquamation of the skin. This type of dermatitis corresponds to that usually called "rat pellagra" in the literature. It has recently been described as the "specific type" of dermatitis by György [1934].

(*b*) A general affection of the skin, without swelling or inflammation, in which there is shedding of the hair, the remaining locks becoming matted and bald patches sometimes occurring later, especially over the head and face and on the anterior surface of the fore paws. The eyes acquire a "spectacled" appearance from loss of the eyelashes and surrounding hair; the eyes are usually sunken and a bloody serous fluid exudes from the sore swollen eyelids, which may be stuck together, and also from the orifice of the nostrils. The urine is scanty and concentrated and dribbling occurs with consequent soiling and staining of the abdomen. These appearances agree well with the description given by György of his "non-specific" skin changes.

In the succeeding pages these two kinds of skin affection are referred to as types (*a*) and (*b*) respectively.

In our experience young rats receiving a diet deprived of vitamin B_2 (in the old sense) may develop either the (*a*) or (*b*) type of dermatitis or a combination of both sets of symptoms. The (*b*) condition is the more usual and generally occurs sooner in young rats deprived of vitamin B_2. The animals affected remain much undersized and appear to suffer constitutionally to a severe degree from the vitamin deprivation. When the florid (*a*) symptoms develop, they usually occur later in rats whose general condition is stronger; sometimes they are superposed on the (*b*) symptoms in animals who survive for some time after the latter have developed.

This generalisation is illustrated in Table I, which gives the history of two litters of rats, which developed dermatitis on a "-B_2" ration. Among 16 rats maintained for 9 weeks after weaning on the experimental diet, the generalised (*b*) skin symptoms were present in 13 and to a severe degree in 6 of these, of which 1 died. At the same date only 8 rats showed signs of "florid" (*a*) dermatitis and in 5 of these it was only slight or incipient and the general condition of the rats was relatively good.

RESULTS.

The effect of flavin was tested on rats whose growth had failed on a diet deprived of vitamin B_2 as well as on those suffering in addition from either form of skin trouble and on some in which there was a syndrome combined of both types.

Effect on growth.

The results collected in Table II show that doses of pure lactoflavin and hepatoflavin, of 20–50γ daily, certainly stimulated growth to a small extent (average weekly increase in weight 5–8 g.), in comparison with the previous performance of the rats on the "-B_2" diet. Smaller doses, 6–12γ, of hepatoflavin were less effective; there was no appreciable increase of weight over a period of 4–6 weeks in the case of 2 rats receiving 6γ, and in 5 out of 8 rats receiving 12γ daily. When, however, doses of 20–40γ flavin were supplemented by a small dose of an autoclaved yeast extract (A) (equivalent to 0·5 to 1·0 g. dried yeast) which showed no vitamin B_2 activity after two successive treatments with fuller's earth at p_H 1·0, a rate of growth was attained which approached the normal standard (12–14 g. weekly increase in weight). A small amount of a yeast extract (B) (equivalent to 0·5 g. dried yeast), in which all the known B-vitamins had been destroyed by autoclaving at 120° for 5 hours at p_H 9 (*ca.*), supplemented flavin equally well, and normal growth was also obtained. With smaller doses of hepatoflavin, the supplements appeared to be less effective and the degree of growth seemed to be limited by the size of the flavin dose (see Table II).

The minimum rat day dose of hepatoflavin which was necessary for "normal growth" was 12–20γ, a value somewhat higher than that found by other workers. György [1935] found 7–10γ lactoflavin adequate as a rat day dose; Euler, Karrer and Malmberg [1934] 5–10γ, but their criterion for growth was lower than that adopted by us.

Table II. *Effect of lactoflavin and hepatoflavin on rats whose growth had ceased on a diet deprived of vitamin B_2.*

Vitamin B_1 given as 0·1 ml. daily (≡ 0·5 g. dry yeast) of a concentrate prepared from brewer's yeast by Peters's method (see Chick and Roscoe [1929]; method modified according to Kinnersley *et al.* [1933]).

Supplement A. Extract of brewer's yeast deprived of B-vitamins by treatment with fuller's earth (dose ≡ 0·5 g. dry yeast).

Supplement B. Yeast extract deprived of B-vitamins by autoclaving 5 hours at 120° at p_H 9 (dose ≡ 0·5 g. dry yeast).

Flavin	Daily dose γ	Average weekly increase in weight over a period of 4–6 weeks				Nature of supplement
		(a) Flavin		(b) Flavin + supplement		
		g.	No. of rats	g.	No. of rats	
Lactoflavin	10	5·1	2	12·3	2	A 1 rat; B 1 rat
„	20	5·3	3	11·7	2	„ „
Hepatoflavin	6	2·6	2	5·1	2	A
				7·1	3	B
„	12–16	4·2	6	8·9	4	A
				10·0	2	B
„	20–50	7·8	8	13·8	5	A
				13·8	5	B
				(c) Supplement only*		
				1·1	7	A

* Time of observation 2–10 weeks.

Effect on dermatitis.

Lactoflavin was found to be without any curative effect upon florid dermatitis ("rat pellagra"), a result confirming that obtained by György [1934]. On the contrary, the symptoms were frequently developed after the administration of flavin to rats whose growth had ceased on the "$-B_2$" diet, but which showed no sign of dermatitis. This was observed in 2 rats receiving 10–20γ of lactoflavin and in 6 rats receiving 12–50γ hepatoflavin (see Table III, rats 98, 141).

Of 9 rats showing the (*b*) group of symptoms in various degrees of severity, the administration of flavin was without any effect in six cases, the result was doubtful in one case, there was slight improvement in one and a most dramatic cure was observed in one severe case (rat 147, Table III) after administration of 24γ hepatoflavin daily. Only in 1 rat showing no skin affection did the (*b*) symptoms develop in slight degree after administration of flavin and in 1 rat slight symptoms became more aggravated. These 2 rats received 20 and 10γ lactoflavin respectively daily.

With one or two exceptions therefore, in these experiments, flavin showed no curative effects on either of the two types of skin disorder.[1] When, however, supplement A or B was given in addition, swift and regular cure of the skin condition accompanied the resumption of growth. There was little difference whether the skin lesions were of the (*a*) or (*b*) type, or a combination of both. Some typical histories of such cures, which included observations on 22 rats suffering from type (*b*) and on 7 rats showing the florid type (*a*), are summarised in Table III.

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[1] See footnote, p. 730.

Table III. *Effect of pure lactoflavin with and without supplements on the dermatitis developed in young rats deprived of vitamin B_2.*

Supplement A = Autoclaved yeast extract, treated with fuller's earth at p_H 1·0 to remove vitamin B_2.
,, AC = Supplement A after dialysis.
,, AD = Dialysate from supplement A.
,, B = Yeast extract, autoclaved at 120° for 5 hours at p_H 9–10 to destroy all known B-vitamins.

Diet St. contained carbohydrate as rice starch (60 % on dry wt.); diet MS as maize sugar (60 %).
(For descriptions of (*a*) and (*b*) types of skin affection, see pp. 724, 725).

Curative material								Body weight			
	Daily dose γ	Supplement	Dose in terms of original yeast (dry wt.) g.	Rat No.	Diet	Time previously on "-B_2" diet weeks	Skin symptoms, and general condition at beginning of cure	At beginning of cure g.	Average weekly increase g.	Duration of cure weeks	Result of treatment
Lactoflavin	20	None	—	98	MS	3	None	47	3·9	10	Florid (*a*) dermatitis developed on hind and fore paws and on nose
,,	20	B	0·5	98	MS	—	As in column 12	86	11·5	4	Cure. Skin condition good, dermatitis healed, fur thick but greying
,,	20	None	—	128	MS	4	Slight (*b*) type symptoms. Fur poor, greasy and stained; eyes, conjunctivitis. General condition poor	32	5·6	5	Skin condition more severe, in spite of some increase in weight
,,	20	A	0·5	128	MS	—	As in column 12	60	9·8	4	Incomplete cure. Skin and fur almost normal, eyes slightly sunken and secreting
Hepatoflavin	12	None	—	141	MS	13	Skin symptoms slight. Eyes sunken and secreting. General condition not good	97	2·0	4	Skin condition deteriorated. Ear tips thickened, paws showing incipient florid (*a*) dermatitis. Wrists stained, eyes sunken and secreting
,,	12	AC	1·0	141	MS	—	As in column 12	105	−2·0	3	Condition much worse in every particular. Severe florid (*a*) dermatitis
,,	12	AD	1·0	141	MS	—	As in column 12	99	6·2	(4)	Cure. Fur much improved, dermatitis healing
,,	12	B	0·5	202	St.	4	Skin symptoms (*b*) type. Fur matted and greasy; bald patches on neck. Condition miserable	36	12·0	4	Rapid cure. Skin and fur became normal in 2–3 weeks, steady increase in weight
,,	12	None	—	181	MS	9	Fur scanty, animal almost bald, eyes very sunken, abdomen stained. Florid (*a*) dermatitis on fore and hind paws	40	2·7	4	Type (*b*) symptoms in eyes, fur improved, florid (*a*) dermatitis present on paws
,,	12	A	0·5	181	MS	—	As in column 12	51	11·7	4	Cure. Florid (*a*) dermatitis healed
,,	24	None	—	147	St.	18	Very severe symptoms of (*b*) type. Fur matted and greasy; abdomen much stained with highly pigmented urine; very severe conjunctivitis. Condition wretched	68	10·3	3	Good cure. Great improvement in eyes and fur in 5 days; staining gone in 2–3 weeks

Influence of the composition of the basal diet upon the development and cure of dermatitis.

(*a*) *Type of carbohydrate.* Whether addition of flavin hastened or stimulated the development of "florid" dermatitis ("rat pellagra") seemed to be related to the nature of the carbohydrate in the "-B_2" basal diet. Among 24 rats on which tests were made with daily doses of unsupplemented flavin, 12 were receiving their carbohydrate in the form of rice starch and 12 as maize sugar. The 8 rats which developed florid dermatitis after dosing with flavin included 7 of the latter group and only 1 of the former.

The administration of flavin, as contained in a small daily dose (5 g.) of raw egg-white, to 5 young rats which had ceased to grow on a "-B_2" ration, while showing no skin affection, caused development of a slight degree of florid dermatitis in 3 receiving the maize sugar diet but restored growth without any effect on the skin in 2 receiving the rice starch diet.

(*b*) *Source and dose of vitamin B_1.* A series of experiments in many ways similar to those described above has recently been carried out by György at Cambridge [György, 1935] and these we have been permitted to observe. In György's experiments the florid type of dermatitis was regularly developed on a "-B_2" diet, in which vitamin B_1 was given in pure crystalline form together with flavin, and was as regularly cured when the vitamin B_1 dose was changed to a Peters concentrate from baker's yeast, given in a relatively large dose (daily rat dose 1–2 ml. equivalent to 10–20 g. fresh, or *ca.* 3–6 g. dry, yeast).

We, on the other hand, had never detected the slightest curative or preventive effect for rat dermatitis in Peters's vitamin B_1 concentrate prepared in our laboratory from brewer's yeast (see Chick and Roscoe [1929], method modified according to Kinnersley *et al.* [1933]), but our rat daily dose (always controlled in separate trials for adequacy in vitamin B_1) was much smaller when reckoned on the basis of the original yeast from which the concentrate was prepared (*i.e.* equivalent to 0·6 g. dry yeast). For this reason alone it might be assumed to carry only about 1/5–1/10 of the adherent yeast "impurity" given in György's dose. It therefore seemed probable that Peters's concentrate, as used by him, might carry an adequate supply of the necessary supplement for flavin. By the kind co-operation of Dr György we have been enabled to test this assumption.

Two rats, 169 and 205, were chosen which had ceased to grow on our "-B_2" diet; both showed severe symptoms of the type (*b*) and one (169) of the florid dermatitis, type (*a*), also. In place of their previous vitamin B_1 addition they received the dose advised by György of his own Peters's vitamin B_1 concentrate (2 ml. $\equiv$ *ca.* 6 g. dry yeast), together with 12γ hepatoflavin. In both cases growth was immediately restored (average weekly increase in weight 18 and 17 g. respectively) and there was swift and complete cure of the skin lesions. For comparison, the effect of a much increased dose (0·3 ml. $\equiv$ 1·8 g. dry yeast) of our Peters's vitamin B_1 preparation, together with 12γ hepatoflavin was tried on 2 litter-mates (rats 167 and 203) of the above rats, which showed a comparable condition of vitamin B_2 deficiency. The effect on growth and dermatitis was very slight in comparison with that observed in rats 169 and 205 and about comparable with that observed when a much reduced dose was given (0·5 ml. $\equiv$ *ca.* 1·5 g. dry yeast) of György's preparation (rat 203). These results are summarised in Table IV.

György [1934; 1935] has interpreted these results as showing a separate specific action for the two types of dermatitis to be possessed by the two con-

Table IV. *Influence of vitamin B_1 ration.*

Diet St. contained carbohydrate as rice starch (60 % on dry wt.); diet MS as maize sugar (60 %).
(For descriptions of (*a*) and (*b*) types of skin affection, see pp. 724, 725.)

Curative material (vitamin B_1 dose)								Body weight			
	Daily dose γ	Supplement	Dose in terms of original yeast (dry wt.) g.	Rat No.	Diet	Time previously on "-B_2" diet weeks	Skin symptoms and general condition at beginning of cure	At beginning of cure g.	Average weekly increase g.	Duration of cure weeks	Result of treatment
Hepatoflavin	12	György's B_1 concentrate (Peters's method)	5·0	169	St.	9	Florid (*a*) dermatitis on ears, hind and fore paws. No conjunctivitis or staining of fur. Condition poor	57	17·7	3	Rapid cure. Marked clearance of symptoms in 10 days
,,	12	,,	5·0	205	St.	8	Skin affection (*b*) type. Fur matted and stained on abdomen	35	11·0	3	Rapid cure. Great improvement in 10 days
,,	12–24	Lister B_1 concentrate	0·6	207	MS	7	Very severe (*b*) type. Fur stained and matted, eyes sunken and secreting, condition miserable	41	2·0	4	Improvement in (*b*) symptoms but eyes still sunken. Development of florid (*a*) dermatitis on ears, fore and hind paws; skin dry, scurfy and cracking
,,	12–24	Gyorgy's B_1 concentrate	1·0	207	MS	—	As in column 12	47	6·2	3	Cure almost complete
,,	12	Lister B_1 concentrate	1·8	167	MS	9	Combined (*a*) and (*b*) types. Ears and paws inflamed, fur stained and matted	52	9·0	4	Incomplete cure. Ears and hind paws not quite normal. Fur much improved
,,	12	,,	1·8	203	St.	8	Combined (*a*) and (*b*) types. Eyes affected, fur greasy and stained, ear tips inflamed. Condition miserable	35	6·3	4	Incomplete cure. Coat improved but paws scurfy. Condition still poor
,,	12	György's B_1 concentrate	5·0	203	St.	—	As in column 12	60	11·0	3	Skin condition improved

stituents of vitamin B_2. Thus "vitamin B_6" (as contained in vitamin B_1 preparations made from yeast by the Peters process) has a definite action in curing the florid (or "specific") dermatitis and in counteracting the influence of flavin in producing this type. Flavin, on the other hand, possesses a definite curative action for the generalised "non-specific" dermatitis. To maintain steady growth both constituents are required.

We also have found the addition of flavin to a diet deficient in vitamin B_2 frequently conducive to the development of the florid type of dermatitis or to its aggravation when present, and we have found further that this action in some way depends on the type of carbohydrate contained in the basal diet. We conclude, however, that in combination with some dialysable, thermo- and alkali-stable substance contained in a yeast extract freed from all known B-vitamins, lacto- or hepato-flavin possesses curative properties for all forms of dermatitis and for the restoration of growth. The supplementary substance derived from yeast would appear to be absent from, or present in only negligible amount in, our Peters's vitamin B_1 concentrate and to be present in relative abundance in György's preparation. It is not possible to state whether this difference is to be explained by the differences in origin and method of preparation of the two products, ours from brewer's and his from baker's yeast, or whether it may not be due to the much larger dose given in his experiments, but the latter explanation appears at least probable.

We have observed the curative action recorded by György of unsupplemented flavin for the "non-specific" (*b*) skin symptoms, but rarely (Table III, rat 147), but we have noticed a definite effect in restoring growth which appears to be proportional to the dose of flavin given.[1]

In our trials with the supplementary material in the absence of flavin, we have obtained neither restoration of growth (see Table II *c*) nor consistent cure of either type of dermatitis. The action of a daily dose of supplement A (equivalent to 0·5 or 1·0 g. dry yeast) was tested over periods varying from 2 to 10 weeks on 7 rats showing skin affections in varying degree. Six of these rats showed the (*b*) type; in 1 of these the symptoms remained unchanged and in 5 definitely progressed. The (*a*) florid type was present to a slight degree in 5 of the rats and absent from 2; in 1 of the latter it developed and among the former the symptoms became worse in 1, remained unchanged in 2 and cleared up in 2.

Previous work by two of the present authors (undertaken to investigate the heat- and alkali-stability of vitamin B_2) showed complete failure of a yeast extract, after autoclaving at 120° for 5 hours at p_H 9·0 (*i.e.* supplement B), to influence dermatitis in rats deprived of vitamin B_2. In almost all the cases treated, the skin lesions were of the (*b*) type [Chick and Copping, 1930, 1; see also Roscoe, 1933]. One case is, however, quoted [Chick and Copping, 1930, 1, p. 934] in which the preparation showed a curative action for the florid type of dermatitis.

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[1] *Note added February 26th, 1935.* Since the above was written we have observed more frequently a curative action of unsupplemented hepatoflavin for the (*b*) type of skin symptoms developed in rats deprived of vitamin B_2. Six rats, of body wt. 39–89 g., showing the (*b*) symptoms (4 in a severe degree) received daily 12 hepatoflavin; all showed some improvement, and 4 were completely cured, with a (subnormal) stimulation of growth. In 3 of the above rats the (*a*) florid type of dermatitis developed during this period.

When the supplementary material alone (vitamin B_6) was given in similar cases, the condition always deteriorated, nor have we observed any consistent curative action of vitamin B_6 for the florid (*a*) type when this was present, unless flavin was given also.

The above results thus contain suggestions that the two constituents of vitamin B_2 may sometimes function separately in the prevention and cure of the two different types of skin disorder, *viz.* flavin for the (*b*) type and the supplementary material for the (*a*) type. To this extent our conclusion confirms that arrived at by Dr György, but the general trend of our whole work indicates that for successful treatment of either condition, or of both when present together, and for maintenance or restoration of growth a combination of both the constituents of vitamin B_2 is necessary. All the above trials were, however, of the curative type and have the defects of this kind of experiment. It is probable that the relation between the two types of skin disorder and the two constituents of vitamin B_2 could be more satisfactorily investigated in prophylactic experiments made to observe the type of skin lesions developed when either flavin or the supplementary material is alone added to the basal "$-B_2$" diet from the time of weaning. Such experiments are now in progress.

Relation of the supplementary substance to the "factor Y" of Chick and Copping [1930, 2].

The facts recorded in the present paper may be summarised in the statements:

(*a*) that flavin is one constituent of vitamin B_2, as previously defined (p. 722), and

(*b*) that the second supplementary constituent is a water-soluble, dialysable, heat- and alkali-stable substance contained in yeast extract.

Vitamin B_2 as contained in natural foods (yeast, whey, liver, egg-yolk) would appear to be an association of these two constituents. In egg-white, however, the supplementary substance appears to be present in relatively small amounts.

In previous observations [Chick and Copping, 1930, 2; Roscoe, 1930] on the growth of rats maintained over a long period on synthetic diets in which the vitamin B_2 was derived from egg-white, and vitamin B_1 given as a minimum dose of a Peters concentrate from yeast, or as Jansen and Donath's adsorbate from rice polishings, a gradual failure of growth always occurred. This could be checked by substituting for the egg-white another natural source of vitamin B_2 or by addition of a yeast extract freed from known B-vitamins by prolonged autoclaving in alkaline solution. The required supplement was present in yeast, liver and egg-yolk, but relatively deficient in egg-white; it was heat- and alkali-stable and was provisionally called "factor Y". Some small amount of this factor was evidently present in our Peters's vitamin B_1 concentrate from brewer's yeast, for when large doses were used they supplemented the action of the vitamin B_2 from egg-white. "Factor Y" would thus appear to be identical with György's vitamin B_6, the supplementary substance needed by flavins in order to exercise the full function hitherto attributed to a single vitamin B_2. Until, however, the biological activity of the flavins was discovered [Kuhn *et. al.*, 1933] the status of our "factor Y" could not be more precisely defined.

Properties of vitamin B_6. Vitamin B_6 or "factor Y" is the most heat- and alkali-stable constituent of the B-group of vitamins; it can resist prolonged autoclaving in alkaline solution (p_H 9·0), *e.g.* in supplement B. For further investigation supplement A was chosen as providing the purer material, but the work has not as yet progressed very far. The activity is found present in the dialysate after dialysis through a cellophane membrane and is not removed by precipitation with lead acetate either at p_H 4·0 or 8·0, in this property affording a contrast with flavin.

Nomenclature of vitamin B_2 and its constituents.

Elvehjem and Koehn [1934], on the basis of experiments with chickens, have suggested that the term vitamin B_2 be reserved for the dermatitis ("pellagra")-preventing factor of vitamin B_2-containing materials. They found that flavins prepared from a liver extract were inactive, but that the fraction remaining after these were adsorbed by fuller's earth was highly active, in "prevention of pellagra". The material they desire to call vitamin B_2 would appear to correspond with our vitamin B_6 or "factor Y". This, from the investigation described in the present paper, is clearly only one constituent of vitamin B_2 as previously understood, and requires to be associated with flavin in order to show the full activity of that vitamin. It seems to us that the use of the term "vitamin B_2" to signify either the non-flavin or the flavin constituent of what in the past was known as vitamin B_2 can only cause confusion in the literature of this subject. The custom adopted by workers concerned primarily with the chemistry of the flavins of alluding to these pigments as "vitamin B_2" adds to the confusion [*e.g.* Kuhn and Weygand, 1934].

We would suggest that the term vitamin B_2 be retained for the association of (i) flavin and (ii) supplementary substance, that (i) be known simply as flavin, since it has been identified with a substance of known chemical constitution, and that (ii) be called vitamin B_6 until such time as its chemical nature and identity are established.

SUMMARY.

1. The results of experiments on rats described in this paper indicate that vitamin B_2, as previously defined, is composed of two constituents: (i) flavin, (ii) a supplementary substance contained in yeast extract.

2. The supplementary substance appears to be identical with the "factor Y" previously described by Chick and Copping [1930, 1], and with the "vitamin B_6" described by György [1934]. It is heat- and alkali-stable, can dialyse through a cellophane membrane, is not adsorbed by fuller's earth at p_H 1·0 or precipitated by lead acetate at p_H 8·0 or 4·0. It is present in small amount in the vitamin B_1 concentrate prepared from yeast by Peters's process and can be detected when large doses are given.

3. Pure hepatoflavin or lactoflavin, when added as a small daily dose (12–20γ) to a basal diet deprived of vitamin B_2, but containing vitamin B_1 (as a minimum daily dose of a concentrate prepared from brewer's yeast by Peters's process), was found to restore growth in rats to a small extent (increase of weight up to 6 g. weekly). In order to attain normal growth (10–12 g. weekly) a small daily dose of the supplementary material (equivalent to 0·5–1·0 g. dry yeast) was also necessary.

4. Flavin when given alone (in doses of 12–20γ daily) was found to be without effect upon the characteristic "florid" dermatitis, type (*a*), "rat pellagra", developed in young rats deprived of vitamin B_2; in some instances its administration appeared to encourage the development of this type of skin disorder in animals where it had not yet developed. In some animals suffering from the more generalised type of skin disorder, described as type (*b*) in this paper, flavin appeared to have a curative action; in others there was no effect.

5. The supplementary material, when given alone (in doses equivalent to 0·5–1·0 g. dry yeast), had no effect in restoring growth, while the (*b*) type of skin disorder, if present, progressed without any check. Upon the florid (*a*) type

of dermatitis, there appeared sometimes to be a slight curative effect, but the results were irregular and inconclusive.

6. When flavin and the supplementary material (in the above doses) were given together, there was a speedy cure of either the (*a*) or (*b*) type of skin disorder, or of both, in cases where there was a combined syndrome, and normal growth was immediately restored.

7. It is suggested, in order to prevent confusion in the literature of the subject, that the term *vitamin* B_2 be retained to signify the two factors in association, that the one constituent be termed simply *flavin*, and the second, supplementary, substance be called *vitamin* B_6, as suggested by György [1934].

In conclusion we wish to express our thanks to Sir Charles Martin for his interest and kindly criticism and to Dr P. György for providing us with materials used for experiments summarised in Table IV, and for his friendly co-operation. Our thanks are also due to the Corn Products, Ltd. for supplying the information regarding the sample of maize sugar used in the basal diet. Our indebtedness to Dr Ph. Ellinger and Dr S. Smith for the samples of pure flavin has already been expressed.

NOTE ON THE INFLUENCE OF LIGHT UPON VITAMIN B_2.

Pure flavin has been shown to be sensitive to visible light, the change being accompanied by loss of biological properties [György *et al.*, 1933]. Many observers in the past have reported destruction of vitamin B_2 in yeast and yeast extracts by exposure to ultra-violet radiation from a quartz mercury arc; and this method has been used to free yeast extracts from vitamin B_2 in the preparation of vitamin B_1 concentrates [Hogan and Hunter, 1928; Hogan and Richardson, 1932]. We, however, found partial destruction of both B-vitamins to occur when a yeast extract was exposed to ultra-violet radiation [Chick and Roscoe, 1929]. It is curious that during many years of vitamin B_2 investigation, we, in common with other workers, should have had no suspicion that this vitamin was sensitive to visible light. The materials used were subject to no special precaution, except that, as a routine, they remained in a dark refrigerator except when being handled. It is probable that, being chiefly yeast products of a brown colour, they were protected by this circumstance. Vitamin B_2 preparations from egg-white [Chick, Copping and Roscoe, 1930] are greenish yellow in colour and here sensitiveness of the vitamin to visible light was demonstrated in the following manner in some recent experiments.

A specimen (EW I) prepared in the ordinary way was found to be unusually poor in vitamin B_2 action and it was then realised that, owing to a change in arrangements, the preparation had been carried out in the month of July on a sunny bench under a window facing south-west. Ordinarily such preparations had been made in a north room and evaporated in a dark cupboard. Another preparation (EW II) was then made, in which the whole process, as far as possible, took place in the dark; this preparation proved to be unusually potent in vitamin B_2 action. The potency of EW I was much enhanced when a supplementary dose (20γ daily) of pure hepatoflavin was given; flavin, on the other hand, in no way enhanced the action of EW II. Addition of Supplement B (alkaline autoclaved yeast extract) increased the growth of rats receiving EW II, but did not assist the action of EW I. These facts indicated that it was the flavin constituent of the vitamin B_2 in EW I that had been destroyed by the sunlight, and that the growth-promoting power of this preparation, even when supplemented by Supplement B, was limited by the small amount of undestroyed flavin present.

REFERENCES.

Chick and Copping (1930, 1). *Biochem. J.* **24**, 932.
—— —— (1930, 2). *Biochem. J.* **24**, 1764.
—— —— and Roscoe (1930). *Biochem. J.* **24**, 1930.
—— and Roscoe (1928). *Biochem. J.* **22**, 790.
—— —— (1929). *Biochem. J.* **23**, 504.
Ellinger and Koschara (1933). *Ber. deutsch. chem. Ges.* **66**, 315; 808; 1411.
Elvehjem and Koehn (1934). *Nature*, **134**, 1007.
Euler, Karrer and Adler (1934). *Ark. Kemi, Min. Geol.* **11** B, No. 33.
—— —— —— and Malmberg (1934). *Helv. Chim. Acta*, **17**, 1157.
György (1934). *Nature*, **133**, 498.
—— (1935). *Biochem. J.* **29**, 741.
—— van Klaveren, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 236.
—— Kuhn and Wagner-Jauregg (1933). *Klin. Woch.* **12**, 1241.
Hogan and Hunter (1928). *J. Biol. Chem.* **78**, 433.
—— and Richardson (1932). *Univ. Missouri Agric. Exp. Stat. Res. Bull.* No. 178.
—— —— (1934). *J. Nutrition*, **8**, 385.
Karrer, Salomon and Schöpp (1934). *Helv. Chim. Acta*, **17**, 419.
Kinnersley, O'Brien, Peters and Reader (1933). *Biochem. J.* **27**, 225.
Kuhn, György and Wagner-Jauregg (1933, 1). *Ber. deutsch. chem. Ges.* **66**, 317.
—— —— —— (1933, 2). *Ber. deutsch. chem. Ges.* **66**, 576.
—— —— —— (1933, 3). *Ber. deutsch. chem. Ges.* **66**, 1034.
—— Rudy and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1950.
—— and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1577.
—— and Weygand (1934). *Ber. deutsch. chem. Ges.* **67**, 2084.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.
Roscoe (1927). *J. Hyg.* **27**, 103.
—— (1930). *Biochem. J.* **24**, 1754.
—— (1933). *Biochem. J.* **27**, 1533.
Warburg and Christian (1932). *Biochem. Z.* **254**, 438.
—— —— (1933). *Biochem. Z.* **266**, 377.

LXXXV. EFFECT OF DEFICIENCY OF VITAMIN B COMPLEX ON THE "REDOX" SYSTEM IN THE EYE-LENS.

By SURENDRA NATH RAY,[1] PAUL GYÖRGY AND LESLIE JULIUS HARRIS.[2]

From the Nutritional Laboratory, Medical Research Council and University of Cambridge, and Department of Physiology, University of Cambridge.

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Previous papers from the Nutritional Laboratory have demonstrated the presence of vitamin C in the lens of the eye [Birch and Dann, 1933; 1934] and this result has since been corroborated by many others [Euler and Martius, 1933; Müller, 1933]. Euler and Martius also made the further interesting observation that, in cataract in man, ascorbic acid could not be detected in the lens. At about the same time Langston and Day [1933] reported that they had been able to produce cataract in rats by diets deficient in vitamin B_2. These two findings suggested that there might be some interrelation between the vitamin B complex and ascorbic acid and other redox systems in the metabolism of the lens, and the present investigation was undertaken with the object of testing this possibility.

The procedure adopted was to keep groups of rats on diets lacking in one or more of the various vitamins of the B group. After a certain time, when the rats had developed lesions characteristic of the particular deficiency, they were killed and their lenses examined for vitamin C by titration against standard 2:6-dichlorophenolindophenol. In certain cases the lenses of animals cured by the administration of the appropriate vitamins were also examined.

Experimental.

The animals were usually killed by coal gas and their eye-balls removed without delay. The eye-balls were carefully incised with a sharp scalpel and the lenses were gently pressed out. To remove the aqueous and vitreous humours surrounding them, the lenses were rolled on a piece of filter-paper. With the application of a little pressure, the softer material could be rubbed away, leaving the harder portion behind. This was used for the estimation of vitamin C.

The small size of the eye-lens made it difficult to estimate the amount of vitamin C by direct titration. For this reason a modification of the micro-titration method of Birch *et al.* [1933] was used. The lenses from the eyes of one rat were weighed and ground up with 0·2 ml. of 20 % trichloroacetic acid and a little washed sand, and the resulting mixture was made up to 1 ml. The solution was centrifuged and an aliquot part (0·3 ml.) of the supernatant liquid was added to a known volume (0·05 ml.) of 2:6-dichlorophenolindophenol solution. The excess of the dye was then back-titrated by a solution of pure ascorbic acid (a solution of 5 mg. of the acid in 100 ml. of 10 % acetic acid solution being generally employed). The dye solution (a 0·1 % solution by weight was usually taken) alone was next titrated against the ascorbic acid solution and the difference gave the amount of ascorbic acid present in the lens extract. With a little practice and when working with lenses from a single normal rat, duplicate results agreed to within 10 %.

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[1] Sir Tarak Nath Palit Research Scholar of Calcutta University.

[2] Member of Scientific Staff, Medical Research Council.

The following two diets were generally used and will be referred to in the text as diets 1 and 4:

Diet 1	
Extracted caseinogen ...	18
Salt mixture	4
Cod-liver oil	1
Butter-fat	9
Rice starch	68

Diet 4	
Glaxo caseinogen A.B. ...	18
Salt mixture	4
Cod-liver oil	1
Butter-fat	9
Cane sugar	66
Agar	2
Irradiated yeast 0·1 g. daily	

Diet 4 is partially deficient in vitamin B complex, and diet 1 is vitamin B-free. In the case of rats on diet 1, the B vitamins were supplied, when necessary, in the following forms: vitamin B_1 as solutions of pure crystalline B_1 (3 pigeon doses a day), vitamin B_2 complex as pure crystalline flavin *plus* "Peters's eluate" [Kinnersley *et al.*, 1933] for antipellagra factor. No supplements were generally used with diet 4, the irradiated yeast being prepared by exposure of dried yeast in thin layers to a mercury-vapour lamp for 24 hours. Fuller experimental details regarding the preparation of experimental animals are given in an accompanying paper [György, 1935, 2]. Adult rats kept on the stock basal diet used in the Nutritional Laboratory (brown bread, milk, green vegetables, with meat and liver once a week) were taken as normal controls.

Results.

The indophenol-reducing capacities (expressed in terms of concentration of ascorbic acid) of the lenses of rats on normal diet, on diet 4 and on diet 1 (supplemented by certain vitamins of the B group) are given in Table I.

Table I.

No. of rat	Wt. of eye-lens (g.)	Conc. of ascorbic acid (mg./g.)	Diet used	Nature of known deficiency
1	0·047	0·53	Normal stock diet	—
2	0·059	0·51	,, ,,	—
3	0·036	0·41	,, ,,	—
4	0·044	0·37	,, ,,	—
5	0·042	0·38	,, ,,	—
6	0·046	0·40	,, ,,	—
7	0·041	0·10	Diet 1 + vit. B_1 + 8γ of flavin + 2 g. of salmon per day	—
8	0·038	0·09	,, + vit. B_1 + 8γ of flavin + 2 g. of haddock per day	—
9	0·029	0·00	,, + vit. B_1 + 8γ of flavin	P-P
10	0·014	0·00	,, + ,, + antipellagra factor	Flavin
11	0·036	0·13	,, + ,, + ,, ,,	
12	0·045	0·08	,, + ,, + ,, ,,	
13	0·027	0·06	Diet 4	Partial deficiency of vit. B_1 and B_2 complex
14	0·041	0·06	,,	
15	0·036	0·14	,,	
16	0·047	0·07	,,	
17	0·041	0·05	,,	
18	0·041	0·13	,, + flavin + antipellagra factor	—
19	0·038	0·00	,, + 40 mg. of acid clay daily for the last 12 days	Partial deficiency of vit. B_2 complex

It will thus be seen that although there is a large quantity of reducing agent (vitamin C) in the lens of the normal rat, the amount present in the lens of a rat kept either on diet 1 or 4 is very low. But in addition it was found in the case of some of the rats kept on diet 4, that when the extract of the lens was added to a measured volume of 2:6-dichlorophenolindophenol, the resulting solution required a larger volume of ascorbic acid solution for complete decoloration than that necessary in control tests for the decoloration of the dye solution alone. This points to the presence of some oxidising substance in these lens. A few such results are given in Table II.

Table II.

No. of rat	Wt. of eye-lens (g.)	Amount of ascorbic acid in mg. oxidised by 1 g. of eye-lens
20	0·037	0·40
21	0·030	0·17
22	0·033	0·25

Significance of the diminished reduction titre.

This diminution of the indophenol-reducing agency almost to vanishing point in the lens of the rat may occasion surprise, since previous experience has shown conclusively that the rat is able to synthesise its own vitamin C [Parsons, 1920; Harris and Ray, 1933]. On the other hand other tissues, such as liver or kidney, from these same animals were found on examination to contain normal amounts of vitamin C. This might suggest either that the lens has a mechanism for synthesising vitamin C different from that present in liver or kidney, or that the vitamin C is not synthesised in the eye-lens but is supplied from elsewhere in the body and that the mechanism of this supply may break down in animals restricted to these special diets. An objection to the last theory may however be raised in the fact that when high doses of vitamin C (50 mg.) were injected into a rat kept on diet 4 its eye-lens showed a slight increase in concentration (about 0·15–0·20 mg./g.) of ascorbic acid. A third and more plausible theory is that the ascorbic acid in the lens forms, with some oxidising agent, part of a complex oxidation-reduction system. By the titration method we are examining only the amount of ascorbic acid in the reduced condition. It may be supposed that when animals are subjected to these special diets, the amount of oxidising substances in the eye-lens increases, thereby converting an equivalent amount of ascorbic acid into some oxidised product. Thus although the amount of total ascorbic acid (reduced + oxidised) may remain the same, it is evident that the titre would fall, thereby giving a false impression of a diminution in the amount of vitamin C. It must be pointed out however that under normal conditions the reducing titre for the lens (ox) is in fact equal to the vitamin C content as determined biologically [Birch and Dann, 1934]. Further work to elucidate these points is now being carried out.

Influence of individual dietetic factors.

The exact dietetic origin of this fall in the total reducing capacity of the eye-lens remains obscure. That inanition is not the cause is shown in Table III. The animals in these experiments were kept on a diet deficient in "vitamin H" [György, 1931; 1935, 1]. Besides loss of weight, they had all developed lesions characteristic of this deficiency. Yet it will be seen that the vitamin C content of the lens is quite normal.

Table III.

No. of rat	Initial wt. (g.)	Final wt. (g.)	Wt. of eye-lens (g.)	Concentration of ascorbic acid in mg./g.
23	76	69	0·040	0·53
24	73	48	0·040	0·45
25	69	57	0·015	0·44
26	73	58	0·026	0·48
27	89	77	0·022	0·49
28	71	55	0·025	0·48

These results demonstrate at the same time that "vitamin H" deficiency is not the cause of this diminution in reducing capacity. Nor does a deficiency of either flavin or the antipellagra factor appear to be responsible; for, as shown in Table I, even when these vitamins are supplied in the diet (Rats 7–12, 18), there is no improvement in the amount of vitamin C. The results with rats 7–12 and 19 also seem to rule out the possibility of vitamin B_1 deficiency being the cause. This conclusion is further brought out in Table IV where the experimental rats were kept on a diet deficient in vitamin B_1 only (alkaline autoclaved marmite being used to provide vitamin B_2 complex). All the rats exhibited advanced polyneuritic symptoms.

Table IV.

No. of rat	Wt. of eye-lens (g.)	Concentration of ascorbic acid in mg./g.
29	0·017	0·31
30	0·018	0·30
31	0·021	0·32
32	0·020	0·20
33	0·017	0·29*
34	0·020	0·27*

* Cured by administration of 1·5 pigeon doses of crystalline vitamin B_1 solution.

It will be noticed that the concentration of the reducing agent in the lens is fairly high and further that no change results when a cure is effected by an injection of vitamin B_1.

A few lenses from vitamin A-deficient rats, kindly provided by Dr W. J. Dann, were also examined, and they were also found to contain normal amounts of reducing factor.

The results seem to show that this peculiar metabolic disorder in the lens of rats subjected to certain diets is probably due to deficiency of some factor which is not identical either with vitamins B_1, B_2 (flavin or antipellagra factor), "H" or A. We have no evidence as yet as to the probable nature of this factor, but while testing several natural foodstuffs, we found raw egg-white to be a potent source of it. Liver extracts as well as fresh liver were also tested but were found to give rather irregular results. The reason for this is not known. The results are shown in Table V.

Table V shows that raw egg-white, administered in daily doses of 5 ml. possesses strong prophylactic as well as curative action. Smaller doses, 3 and 2 ml. per day, were also tried but the results then were not so clear-cut and gave rather variable values. It may also be pointed out that rats nos. 35–38 were suffering from acute dermatitis owing to deprivation of the antipellagra factor, yet the high reducing capacity of the lens emphasises the the non-identity of the antipellagra and eye factors.

Table V.

No. of rat	Wt. of eye-lens (g.)	Conc. of ascorbic acid in mg./g.	Diet used
35	0·030	0·39	Diet 1 + 5 ml. egg-white daily + vit. B_1
36	0·031	0·32	,, + ,, ,, + ,,
37	0·038	0·28	,, + ,, ,, + ,,
38	0·029	0·23	,, + ,, ,, + ,,
39	0·034	0·37	,, + ,, ,, + ,, + haddock (antipellagra factor)
40	0·028	0·43	,, + 5 ml. egg-white daily + vit. B_1 + Peters's eluate
41	0·034	0·22	Diet 4 + 5 ml. egg-white daily for the last 15 days
42	0·036	0·30	,, + ,, ,, ,,
43	0·021	0·25	Diet 1 + 2 g. liver daily
44	0·041	0·00	,, + ,, ,,
45	0·035	0·37	,, + B_1 + flavin + 2 ml. liver extract daily
46	0·047	0·11	,, + ,, + flavin + 2 ml. liver extract daily + Peters's eluate
47	0·047	0·20	,, + ,, + flavin + 2 ml. liver extract daily + Peters's eluate

It is as yet too early to suggest whether this diminution of the reducing power of the lens is due to some new vitamin. The nature of the factor present in raw egg-white is being investigated. We also hope to publish at a later date an account of the histological changes, if any, in the lens of the eye due to deprivation of this factor.

Vitamin C.

The vitamin C content of the eye-lens of guinea-pigs, both normal and scorbutic, has also been examined. As may be imagined, the vitamin C in the lens appears to depend upon the amount given in the food but curiously enough quite an appreciable amount of the vitamin (about 0·2 mg./g.) is held back tenaciously by the eye-lens, even when the animal has died from acute scurvy. The effect of chronic scurvy as well as combined deficiency of vitamins B and C is under investigation, and fuller details will be published later.

Oxidising agent present in blood.

According to van Eekelen *et al.* [1933; see also Emmerie and van Eekelen, 1934] vitamin C is present in a reversibly oxidised form in the blood. Direct titrations of trichloroacetic acid extracts of the blood of normal animals against 2:6-dichlorophenolindophenol, indeed, fail to show any reduction. With guinea-pigs or human beings given large doses of vitamin C, extracts of blood show small reducing values which however are almost negligible compared with the amount of vitamin C ingested. Nevertheless when extracts of normal blood were examined by the new back-titration method, the presence of some oxidising agent, of the same nature as that occasionally seen in the lens of rats fed on deficient diets (p. 737), was encountered. This finding suggests that there may be some agency present in blood which is capable of oxidising ascorbic acid, possibly into the reversibly oxidised form. If the amount of ascorbic acid ingested is very great, it is evident that after it has reduced the whole of the oxidising agent in the blood, some of the ascorbic acid may still remain and this surplus will give a net reducing value to the blood. Conversely, an increased concentration of the oxidising agent may be expected to be found in scorbutic animals. Only a few experiments have been performed in this direction and though the results seem to confirm this theory, much additional work is required before anything more definite can be said.

The results are summarised in Table VI.

Table VI.

No. of rat	Nature of animal	Wt. of blood taken (g.)	Amount of ascorbic acid in mg. capable of being oxidised by 1 g. of whole blood
48	Normal	8·65	0·019
49	,,	4·75	0·026
50	Diet 4	3·30	0·022
No. of guinea-pig			
G1	Normal	9·40	0·00
G2	19 days on scorbutic basal diet	8·55	0·039

SUMMARY.

1. A new technique for estimating vitamin C by a method of back-titration is described. By its means the indophenol-reducing capacity of the lens from a single normal rat can readily be estimated, the error of determination being within ± 10 %.

2. The indophenol-reducing capacity of the eye-lens as determined by this method is greatly diminished in rats which have been subjected to certain experimental diets lacking in various constituents of the vitamin B complex. In extreme cases, extracts of the lens, instead of showing a reducing capacity, actually oxidise ascorbic acid. The cause of the abnormality does not appear to be due to deficiency of vitamins B_1, B_2 (either flavin or antipellagra factor), "H" or A. It is however too early to say whether a new vitamin is involved.

3. Raw egg-white possesses marked prophylactic and curative action in this disorder.

4. The blood of normal and of deficient animals contains an oxidising agency similar to that found under certain conditions in the lens.

REFERENCES.

Birch and Dann (1933). *Nature*, **131**, 469.
—— —— (1934). *Biochem. J.* **28**, 638.
—— Harris and Ray (1933). *Biochem. J.* **27**, 590.
van Eekelen, Emmerie, Josephy and Wolff (1933). *Acta Brev. Neerl.* **3**, 168.
Emmerie and van Eekelen (1934). *Biochem. J.* **28**, 1153.
Euler and Martius (1933). *Z. physiol. Chem.* **222**, 65.
György (1931). *Z. ärztl. Fortbildung.*
—— (1935, 1). Handbuch der Kinderheilkunde by Pfaundler-Schlossmann (Berlin).
—— (1935, 2). *Biochem. J.* **29**, 741.
Harris and Ray (1933). *Biochem. J.* **27**, 2006.
Kinnersley, O'Brien, Peters and Reader (1933). *Biochem. J.* **27**, 225.
Langston and Day (1933). *Southern Med. J.* **26**, 128.
Müller (1933). *Nature*, **132**, 280.
Parsons (1920). *J. Biol. Chem.* **44**, 587.

LXXXVI. INVESTIGATIONS ON THE VITAMIN B_2 COMPLEX.

I. THE DIFFERENTIATION OF LACTOFLAVIN AND THE "RAT ANTIPELLAGRA" FACTOR.

By PAUL GYÖRGY.

From the Physiological and Nutritional Laboratories, Cambridge.

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By the end of 1932 three separate components had been distinguished in the vitamin B complex as needed by the rat: (1) vitamin B_1, the antineuritic factor; (2) vitamin B_4, the absence of which was said to be associated also with symptoms of nervous lesions such as disturbances of co-ordination and ataxia; and finally (3) vitamin B_2, the antipellagra factor. In contrast with the first two, the relatively high thermostability of vitamin B_2 had been noted as its special characteristic. All the three factors mentioned display growth-promoting properties under suitable circumstances. Some have held that vitamin B_2 is divisible into two further factors [Sure *et al.*, 1931; Thatcher *et al.*, 1931; Gurin *et al.*, 1931], or at least that this is probably the case [Sherman and Sandels, 1929; 1931]. In this fractionation of vitamin B_2, one factor is alleged to be predominantly growth-promoting, the other being the antipellagra factor. However, none of the supporters of this theory has been able to bring forward convincing proof of his views. Roscoe, on the grounds of her wide experience, recently stated [1933, 2] that "no support was found for the theory postulating the existence of separate dietary factors respectively for preventing and curing dermatitis and for promoting growth".

Chick and Copping [1930] showed that an alkali- and heat-stable, water-soluble, growth-promoting factor, called by them factor Y, probably existed, but no experimental work has yet determined its precise position in the vitamin B complex.

The biological analysis of vitamin B_2 was attended with great difficulties. Almost all workers were agreed that the specific symptoms peculiar to vitamin B_2 deficiency, as first described by Goldberger and Lillie [1926], and regarded by them as analogous with pellagra in man, only appeared irregularly in rats [*cf.* Medical Res. Council, 1932, p. 151]. In another recent paper Roscoe [1933, 1] gives "data concerning the incidence of dermatitis among the vitamin B_2-deficient animals observed in the Nutritional Laboratory of the Lister Institute during the past seven years". Skin changes were noted in only 108 out of a total of 191 rats. The average time taken for symptoms to develop was 10 weeks. Roscoe employed the usual vitamin B-free, synthetic diet, supplemented by vitamin B_1 $(+B_4)$ in the form of a concentrate from brewers' yeast prepared according to Peters [*cf.* the new directions of Kinnersley *et al.*, 1933]. Other authors used an alcoholic extract of maize [Goldberger and Lillie, 1926], or of wheat [Bourquin and Sherman, 1931] as the source of vitamin B_1. Dried yeast irradiated with ultra-violet light and liver extracts similarly irradiated [Hogan and Richardson, 1932, 1, 2; 1933] or irradiated rice polishings [Thatcher *et al.*, 1931] are said to be equally suitable forms in which vitamin B_1 may be given in experiments on vitamin B_2. With these dietetic modifications, and using alcoholic extracts of maize and wheat, the percentage of rats deprived of vitamin B_2 which show pellagra-like dermatitis reaches a satisfactorily high level; but even these experimental methods do not yield uniform results.

The object of our own investigations was the study of the skin changes, and the work of Sherman and Sandels, which appeared about the time we were beginning our experiments, suggested to us that this object could be most rapidly and easily attained by the use of the diet described by Bourquin and Sherman [1931].

These experiments have been reported in several papers in association with Kuhn and Wagner-Jauregg [1933; 1934]. In addition to a number of minor observations, they yielded two new and important pieces of information. (1) Vitamin B_2 is in fact not a single substance but may be subdivided into two subsidiary components; and (2) it was possible chemically to identify one of these subsidiary factors, which was first prepared in crystalline form from milk. This natural dyestuff, called lactoflavin, corresponds to the coloured component of the "yellow oxidation ferment" of Warburg and Christian [1932, 1, 2; 1933], and is present, under natural conditions, in foodstuffs, not only in the free state, but also in the form of Warburg's ferment, *i.e.* in combination with a protein carrier as a non-dialysable "flavoprotein" [György *et al.*, 1934, 3].

When the experimental diet of Bourquin and Sherman is used it is not possible to produce an increase in the weight of animals whose growth has become stationary merely by the addition of pure lactoflavin to the diet. The addition of some other factor (or factors) is found to be necessary. Our first active supplements to the basal diet of Bourquin and Sherman consisted of concentrates from yeast from which the flavins had been removed by adsorption on fullers' earth in acid solution. Such solutions by themselves were however completely inactive, and animals, after a slight initial gain in weight, generally remained at a constant weight for weeks on end. If lactoflavin were administered at this juncture there was a dramatic resumption of growth. The active yeast residue referred to above may be replaced by a concentrate of vitamins B_1+B_4 such as may be obtained by adsorption of purified concentrates from yeast on charcoal, and subsequent elution with alcohol containing hydrochloric acid [Kinnersley *et al.*, 1933; György *et al.*, 1933, 1, 2; 1934]. Since however the results of these experiments were independent of the vitamin B_1, which was present in considerable amounts in the diet of Bourquin and Sherman (extract of wheat), we were inclined at first to correlate the indispensable supplementary substance with vitamin B_4.

In the isolation of lactoflavin, and its identification as one of the components of the vitamin B_2 complex, the growth rate was used exclusively as biological criterion and standard. The unit dose was defined according to the rules laid down by Chick and Roscoe [1927], as that quantity of lactoflavin which would give rise to a gain in weight of 10 g./week for at least 4 weeks. Under the experimental conditions mentioned (diet of Bourquin and Sherman *plus* flavin-free supplementary substance), 7–10 γ of lactoflavin were found to be equivalent to 1 rat day dose [György *et al.*, 1933, 1; 1934, 3]. On the other hand, in order to produce the full effect of lactoflavin, we required as supplementary factor a quantity of yeast concentrate, as prepared by Kinnersley *et al.*, equivalent to 5–15 g. of fresh bakers' yeast.

The growth-promoting action is only one property of vitamin B_2, which owes its peculiar position in the vitamin series to its supposed protective action against pellagra. To what extent lactoflavin also possesses this biological property is an important question to which the results already referred to give no answer, for, in our numerous original experiments, we failed to observe a single case of genuine, well-marked, pellagra-like dermatitis. Vitamin B_2 deficiency was expressed only as cessation of growth, followed later by a gradually progressive decrease in weight. Thus an important gap arose. The filling of this gap was the task to which the experiments now described were directed.[1]

In the meantime, the scope of the task had widened considerably. The precise nature of the "supplementary substance" which was present in the vitamins B_1+B_4 concentrates of Kinnersley *et al.* was to be submitted to a more exact analysis. Owing to the inconstant chemical composition, and therefore uncertain nature, of the wheat extract which served as the source of vitamin B_1 in the diet of Bourquin and Sherman, I preferred to employ for my other experiments the crystalline preparation of Windaus *et al.* [1931], or a highly purified vitamin B_1 product, of which 8–12 γ were equivalent to 1 pigeon day dose. I was supplied with both these preparations through the generosity of the I. G. Farbenindustrie, Elberfeld, Germany.

The distribution of vitamin B_2 in animal products has also been re-investigated [see György, 1935, 1], since, owing to the state of our knowledge at the time when our previous investigations were undertaken, the complex nature of vitamin B_2 could not then be taken into account [György *et al.*, 1934, 1]. Finally I have made a renewed attack on the problem of the photosensitivity of vitamin B_2 [György *et al.*, 1933, 2] or of its components, having regard, this time, to the pellagra-like skin changes [see György, 1935, 2].

EXPERIMENTAL.

A. *Use of the diet of Bourquin and Sherman for the production of pellagra-like dermatitis in rats.*

There were several possibilities which might account for the failure to produce pellagra-like skin changes in the experiments performed with Kuhn and Wagner-Jauregg. The relatively short duration of the preliminary depletion period, during which the animals received no supplements in addition to the diet of Bourquin and Sherman, has already been referred to in previous papers. The animals used for the experiments were very young, generally weighing 20–30 g. The weight frequently became stationary after 1 week only. After 4, or at the most 6 weeks, the animals became so weak that treatment with active B_2 concentrates could usually be no longer postponed. In order to demonstrate the essentially supplementary action of flavin, feeding with flavin-free solutions was begun early, usually in the 3rd to 6th week of the experiment. This was done on the assumption that flavin was the actual pellagra-preventing substance. If this assumption were incorrect, and if the pellagra-preventing substance were contained instead in the supplementary factor, then the absence of pellagra-like dermatitis would be the logical result of this experiment. Finally the possibility has to be discussed whether alcoholic wheat extracts vary in pellagra-preventing activity, so that some of them may be able alone to prevent the development of pellagra.

The experiments performed to clear up this possibility gave positive answers to all the questions which have been considered.

Fifteen rats, ranging in weight from 30 to 40 g., were put on a Bourquin-Sherman diet; five of these received no further supplements, five received 10 γ

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[1] A preliminary communication appeared in *Nature*, **133**, 498, March 31st, 1934.

each of lactoflavin daily, and the remaining five animals each received 1 ml. of a solution of vitamins B_1+B_4 [Kinnersley *et al.*, 1933] equivalent to 10 g. of fresh bakers' yeast. After 5–8 weeks, two animals of the first and three of the second group showed definite pellagra-like manifestations of the skin, while the third group remained free from symptoms, their weight however remaining stationary in common with the rats of the other two groups. It chanced that the supply of maize-starch impregnated with alcoholic extract of wheat ran out at this stage of the experiment and a new sample had to be used. The result was a gradual disappearance of the pellagra-like skin changes. In addition, the group of animals treated with supplements of lactoflavin displayed an increase in weight side by side with the improvement in the skin symptoms (Fig. 1). The weight of the animals receiving no lactoflavin remained stationary. It must therefore be concluded that the alcoholic wheat extract used later in the experiment, in contrast to the extract used previously, contained a amount of pellagra-preventing substance which was sufficient to bring about the disappearance of the pellagra-like dermatitis. At the same time another unexpected conclusion probably follows from this observation, namely that lactoflavin is not the pellagra-preventing substance, but that under certain circumstances, large amounts of the pellagra-preventive factor may be present in an alcoholic wheat extract, and that it is also present apparently in the alcohol eluate from charcoal prepared from bakers' yeast [Kinnersley *et al.*, 1933]. Pellagra was cured in all these animals in 7 weeks. The use of untreated rice-starch in place of the maize-starch loaded with alcoholic wheat extract, and the use of a solution prepared from crystalline vitamin B_1 (from the picrolonate) as a source of vitamin B_1 (3 pigeon day doses daily) brought about another immediate cessation of growth of the animals, followed by progressive loss of weight, and after 3–4 weeks, an acute exacerbation of "rat pellagra". This subsequent period gave a further setback to the conclusions which had previously been drawn.

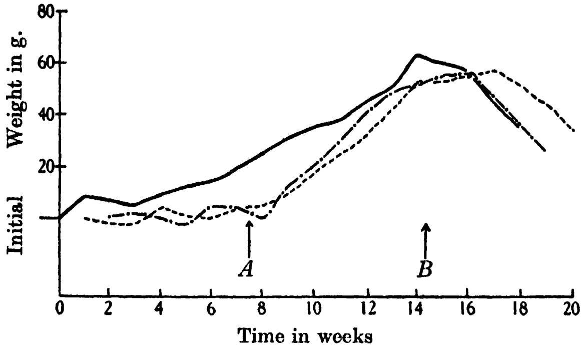

Fig. 1. The weight curves of three rats which received 10γ lactoflavin daily in addition to the diet of Bourquin and Sherman. At *A* the alcoholic extract of whole wheat was replaced by a new sample, and this in its turn was replaced at *B* by a crystalline preparation of B_1. All the animals showed symptoms of pellagra at the end of the experiment.

The results of the experiments just described show that, with a suitable wheat extract, the diet of Bourquin and Sherman may be employed with advantage in experiments on "pellagra". The uncontrollable variations in the contents of "pellagra-preventive" factor (probably depending on the type of wheat used and on the composition of the alcoholic wheat extract) constitute a very disturbing element of uncertainty which may under certain circumstances completely prevent the carrying out of the desired experiments. Accordingly it is recommended that if a crystalline, or similarly well-characterised and highly purified preparation of vitamin B_1, can be obtained the diet of Bourquin and Sherman should be dispensed with in experiments of which the purpose is the study of pellagra-like dermatitis. In addition to this, it must be borne in mind that yet other unknown components of the vitamin B complex may be present in the alcoholic wheat-extract. Thus work on the vitamin B complex, and on the B_2-group in particular, may be made to give quite illusory results.

B. *Use of highly purified or crystalline vitamin B_1 in the analysis of the vitamin B_2 complex.*

By the use of crystalline vitamin B_1 in a diet otherwise free from representatives of the vitamin B group, the analysis of the vitamin B_2 complex became more exact. This advance possesses a certain disadvantage from the biological point of view. For the study of the symptoms of deficiency of any particular component of the vitamin B_2 complex, all the other members of this group must be present in the experiment. It is known from numerous experiences in the study of vitamins that the appearance of the uncomplicated pure, specific, deficiency disease can only be assured if this condition is fulfilled. It is highly probable that the diet of Bourquin and Sherman comes nearer to satisfying this condition than a pure, or highly purified preparation of vitamin B_1. Nevertheless this disadvantage should not be over-estimated, and the attempt at analysing the vitamin B_2 complex should be undertaken by a clear, comprehensive method. The isolation of lactoflavin, with the help of the diet of Bourquin and Sherman, represents an important achievement for the new biological analysis. We now have at our disposal, in the form of crystalline preparations, both vitamin B_1 and lactoflavin. It remained to be shown in separate experiments whether the charcoal eluate obtained from bakers' yeast by the method of Peters was also capable of fulfilling the rôle of supplementary factor in these modified experiments.

The basal diet was constituted as follows:

Caseinogen AB "Glaxo"	18
Rice-starch	68
Butter fat	9
Cod-liver oil	1
Salt mixture (B.D.H.)	4

Caseinogen AB "Glaxo" extracted with alcohol was used in the later experiments, but no definite biological differences could be detected, in these experiments, between the two specimens of caseinogen.

The experimental animals employed were albino and piebald rats of various weights. In the earliest experiments the weights ranged chiefly from 35 to 50 g. The stock diet for one group of rats consisted of milk, bread and wheat kernels, with meat and cabbage once weekly. The majority of the rats were descended from a single stock reared on the following regulation stock diet in use at the Nutritional Laboratory:

Monday.	Bread, milk, cabbage and mixed corn.
Tuesday.	Bread, milk, liver.
Wednesday.	Boiled wheat, milk, yeast and cabbage.
Thursday.	Bread, milk, meat and mixed corn.
Friday.	Bread, milk and carrots.
Saturday.	Bread, milk, cabbage and mixed corn.
Sunday.	Bread, milk and carrots.

Lettuce twice a week when obtainable.

The change in the stock diet was without detectable influence on the time of appearance of pellagra-like skin changes, on their intensity or on the absence of growth which occurs with an experimental diet free from vitamin B_2 complex. Differences in sex or in pigmentation (albino or black-and-white) were also found to be of no significance in these investigations.

In addition to the experimental diet the rats received vitamin B_1, at first in the form of a crystalline preparation (from vitamin B_1 picrolonate) in doses of 3–4 pigeon day doses dissolved in 2 ml. of water daily (Sundays excepted). A lengthy series of experiments showed that a highly purified vitamin B_1 concentrate, 1 pigeon day dose of which was equal to about 8–12 γ of the dry residue, performed the same function as the pure picrolonate. The latter substance, which is more easily prepared, was therefore used in preference. Henceforth every new experimental result was invariably controlled with the crystalline preparation serving as the source of vitamin B_1.

The first task was the repetition of the results previously obtained with the diet of Bourquin and Sherman. The most urgent question was whether the character of lactoflavin, as a component of the vitamin B_2 complex, could be brought out by the modified experimental method also.

To this end the animals were given, together with the preparation of vitamin B_1, Peters's eluate [Kinnersley *et al.*, 1933] from bakers' yeast, such as had already proved of value as a supplementary factor when the diet of Bourquin and Sherman was being used [György *et al.*, 1934, 3]. 1 ml. of a solution, equivalent to 10 g. of fresh bakers' yeast, was given daily (again Sundays excepted). Despite these supplements however the growth of the animals remained almost completely stationary, apart from a slight increase in the first weeks of the experiment. If, after a longer or shorter preliminary period, the animals are given lactoflavin, a striking increase in weight at once sets in which persists with practically no diminution for 4–6 weeks. If the order of the experiment be now reversed and the animals be fed first with vitamin B_1 *plus* lactoflavin, growth will remain stationary in precisely the same way. In fact the inhibition of growth in this case is generally most impressive and is as a rule absolute. In this experiment the subsequent administration of Peters's eluate causes as sudden a resumption of growth as did that of lactoflavin with the animals which were given vitamin B_1 *plus* Peters's eluate in the first place and lactoflavin subsequently. The change in the order of the experiment does not affect the result, at all events so far as the resumption of growth is concerned (Fig. 2).

Thus the information obtained in previous researches [György, Kuhn and Wagner-Jauregg, 1933; 1934] was confirmed once more with the aid of this clear and simplified experimental technique. Vitamin B_2 therefore may be subdivided into two subsidiary factors: (*a*) the crystalline, well-defined lactoflavin and (*b*) the "supplementary factor" (or perhaps supplementary factors) contained in Peters's "charcoal eluate" from bakers' yeast. Growth can only be brought about by the combined actions of both these components, independently of the order in which they are exhibited.

This result is of critical importance, the key as it were to all further attempts at analysing the vitamin B_2 complex. It was confirmed by numerous subsequent experiments. Of these eleven were devoted exclusively to this purpose. In seven of these the animals received Peters's eluate in addition to vitamin B_1 in the preliminary stage. In four others they received lactoflavin only in addition to vitamin B_1. Lactoflavin was given in doses of 7–10 γ daily, and Peters's eluate in amounts equivalent to 10 g. fresh bakers' yeast. The average weekly gain in weight for the eleven experiments was 11 g. in an experimental period of 4 weeks, and 10 g. in a period lasting 6 weeks. It should be emphasised that the variations in the values obtained for individual animals were very slight. Increasing the quantity of lactoflavin to 20 γ or of yeast eluate to the equivalent of 20 g. fresh bakers' yeast did not increase the rate of growth to any notable extent. On the other hand, reduction of the doses, whether of lactoflavin or of yeast eluate, led to an insufficient acceleration of growth.

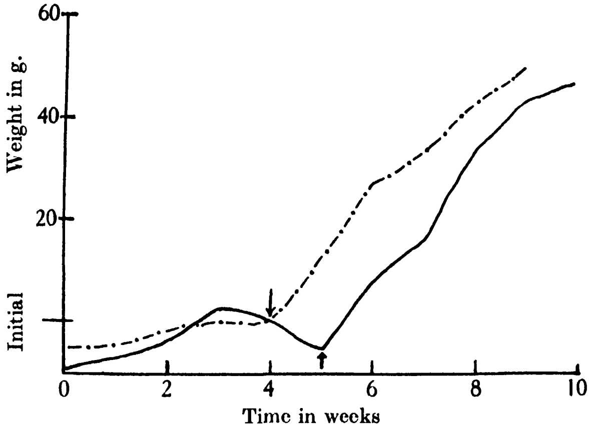

Fig. 2. Apart from the basal diet both animals received 3 pigeon doses daily of vitamin B_1. In addition to this the animal —— received 10γ lactoflavin, and the animal —·— 1 ml. Peters's eluate daily (equivalent to 10 g. of fresh bakers' yeast). At ↑ the animal —— was given 1 ml. of Peters's eluate also, and at ↓ the animal —·— 10γ lactoflavin. An increase in weight was only produced by the combined administration of vit. B_1 + lactoflavin + Peters's eluate.

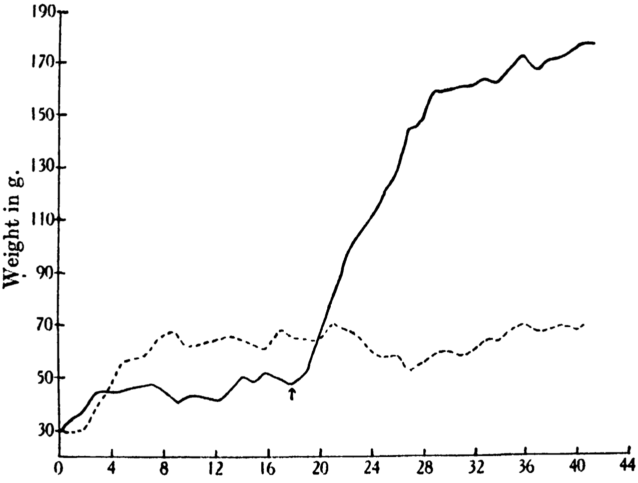

Fig. 3. The weight-curves of two animals which, apart from the basal diet, received at first only vitamin B_1 + Peters's eluate. At ↑ the animal —— was given 10γ lactoflavin daily in addition. The doses of Peters's eluate and lactoflavin were doubled in the 32nd week.

The daily requirements established in this case hold good only for the experiment described. It is quite possible, for example, as Euler *et al.* [1934] have recently emphasised, that the daily requirement for lactoflavin may be lowered if the animals are given all the other components of the vitamin B_2 complex at the same time, or at all events if they are given them in a more favourable combination than in the above experiment. In this respect Peters's yeast eluate does not fulfil the highest requirements as a supplement to lactoflavin. This may clearly be seen from the fact that animals receiving vitamin B_1, lactoflavin and Peters's eluate maintain the normal rate of growth only up to a body weight of 150–170 g. Growth cannot be pushed beyond these limits even by increasing the doses of lactoflavin and yeast eluate. The curve reproduced (Fig. 3) is particularly instructive in this respect.

Crystalline lactoflavin may also be replaced by egg-white.

Animals receiving 3–5 ml. daily of raw egg-white in addition to a basal diet *plus* purified vitamin B_1 react in the same manner as in the experiments with vitamin B_1 and crystalline lactoflavin. Growth remains at a complete standstill and shows no change for weeks on end. Feeding with Peters's preparation at this point promptly brings about the expected increase in weight (Fig. 4). Up to the present we have been unable to find any definite differences between the reactions of animals receiving crystalline lactoflavin and those in receipt of raw egg-white as the source of lactoflavin. The amount of Peters's eluate required in this experiment was again equivalent to 10 g. of fresh bakers' yeast. A dose of 3 ml. of egg-white as the source of lactoflavin is sufficient to maintain the growth at the standard rate. An increase in the dose of egg-white to 5–10 ml. produced no additional increase in growth, nor did an increase in the dose of lactoflavin from 7–10 γ to 20 γ.

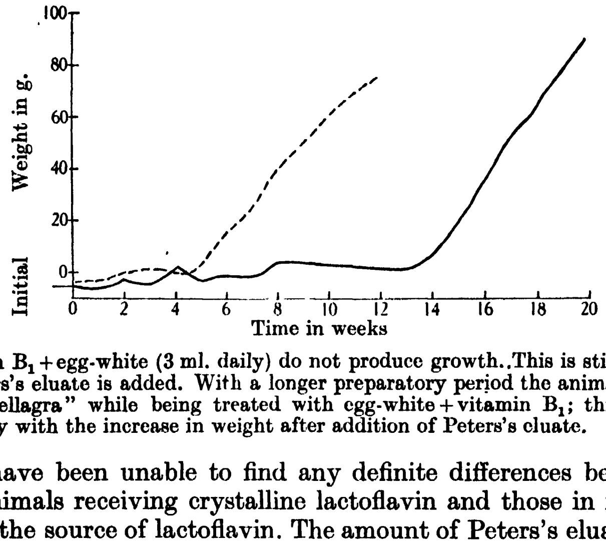

Fig. 4. Vitamin B_1 + egg-white (3 ml. daily) do not produce growth. This is stimulated immediately Peters's eluate is added. With a longer preparatory period the animal —— displayed typical "pellagra" while being treated with egg-white + vitamin B_1; this rapidly healed coincidently with the increase in weight after addition of Peters's eluate.

The experiments which have just been described possess special significance with regard to the question of the existence of vitamin B_4 [Reader, 1929; 1930, 1, 2]. It is worthy of note that rats receiving crystalline vitamin B_1 (3–4 pigeon doses daily) and no further addition of vitamins (with the possible exception of crystalline lactoflavin only) do not exhibit any symptoms which can be taken as specific of vitamin B_4 deficiency (Reader), even in experiments extending over as long as 10–15 weeks and more. Nervous manifestations typical of this condition in particular were absent, such as ataxia, loss of co-ordination and spastic gait.[1]

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[1] The nature of the symptoms held to be due to vitamin B_4 deficiency is discussed in a separate paper by Birch and György in press.

C. *Pellagra-like dermatitis*[1] *and the vitamin B_2 complex.*

The incidental observations made in the experiments with the diet of Bourquin and Sherman, that pellagra-like dermatitis was dependent not on the absence of lactoflavin but on that of the "supplementary factor", was convincingly confirmed by a further extensive series of experiments with the modified technique. In a group of over fifty rats which received purified vitamin B_1 and Peters's eluate in quantities equivalent to 10 g. fresh bakers' yeast, not a single case of skin changes of a pellagra-like character was observed. This negative result cannot be explained by the shortness of the period during which the animals were under observation, for a large number (24) of the experimental animals were treated with vitamin B_1 and Peters's eluate for at least 12 weeks. At the same time the growth curve, after an initial but definite rise, took a horizontal, or a moderately rising course (*cf.* as example Fig. 3). The relevant figures are given in the following table:

No.	Weeks	Increase in wt. (g.)	No.	Weeks	Increase in wt. (g.)
1	19	21	13	16	18
2 +	15	16*	14 +	14	7*
3	20	40	15 +	27	79
4	19	28†	16	23	63
5	20	13	17 +	26	44
6	20	35	18 +	13	10*
7	19	37	19	21	38
8	24	46	20	41	39
9 +	23	17	21 +	21	34
10 +	29	50	22 +	12	6
11 +	18	35*	23 +	20	39
12 +	26	27*	24 +	22	38

* Died. † 33 g. in first 5 weeks and – 5 g. in the remaining 14.

In about one half of the rats submitted to over 12 weeks' experimentation (*i.e.* in 13 marked + out of 24 in the above table) the deficiency of lactoflavin expressed itself in these experiments not only as stationary weight but in certain well-marked associated symptoms, the most important of which were changes in the skin and fur. These latter, however, were fundamentally distinct from the specific "pellagra-like" changes. They differ chiefly in being much less striking. In deficiency of lactoflavin (at least under the experimental conditions selected by us) a gradually progressive loss of hair occupies the foreground of the picture. It is localised to certain areas, such as the back between the shoulders, the lines joining the eyes and ears leaving a median comb of hair remaining in between and finally the chest. In many cases there may be observed on the uninflamed skin, in the midst of the scanty patches of fur, minute, almost punctate whitish-yellow scales. In advanced cases the eyes are sometimes surrounded by a spectacle-like loss of hair, while the eyelids are gummed together. The extremities and the ears invariably escape all changes whatsoever. If treatment, *i.e.* addition of lactoflavin, is not begun in time, the animals succumb to general exhaustion. The end is usually ushered in by meteorism (which is presumably an expression of intestinal atony), by dyspnoea (which is probably due to the last-named symptom and is reminiscent of the laboured breathing seen in pneumonia) and by blue discoloration of the extremities (circulatory failure?). The administration of lactoflavin at the appropriate time will avert this end with absolute certainty. With the resumption of growth all the other associated symptoms of lactoflavin deficiency begin to resolve.

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[1] Throughout this series of papers the term "pellagra", as applied to rats, is used without prejudice as to its identity or non-identity with human pellagra.

While in lactoflavin deficiency the skin changes just described appear irregularly and are not very striking, in rats which received in addition to the basic diet vitamin B_1, or vitamin B_1 *plus* lactoflavin or egg-white, but not the "supplementary factor" contained in Peters's eluate, the skin disorders completely fill the picture. It is here that we encounter the specific symptom-complex of pellagra-like dermatitis first described by Goldberger and Lillie [1926]. This peculiar skin disorder appeared in every case provided the experiments were prolonged for a corresponding length of time. We already possess observations on 211 cases of this nature which we collected during 1934. Of these 55 have had the disease twice, *i.e.* having been successfully treated on the first occasion they again fell ill when submitted to the original conditions once more. The animals may be placed in six groups according to the supplements they received.

Group	Supplement	Total no. of animals	First symptom of "pellagra" after av. no. of weeks	Animals ill twice	First symptom of "pellagra" of second illness after av. no. of weeks
1	Crystalline vitamin B_1 (3–4 pigeon units daily)	10	6	1	7
2	Purified vitamin B_1 preparation (3–4 pigeon units daily)	12	7	3	8
3	Crystalline vitamin B_1 + lactoflavin (10γ daily)	31	6	8	6
4	Purified vitamin B_1 preparation + lactoflavin (10γ daily)	65	7	18	7
5	Purified vitamin B_1 preparation + 3 ml. egg-white daily	59	7	18	8
6	Purified vitamin B_1 preparation + 5 ml. egg-white daily	34	8	7	11
	Total	211	7	55	8

Thus with this experimental technique it has been possible to overcome the element of uncertainty which has hitherto prevailed in the experimental production of pellagra-like dermatitis. Differences in the supplements used did not affect the incidence of the clinical picture of pellagra-like dermatitis. Within narrow limits it was a matter of indifference whether the animals received only vitamin B_1 in addition to the basal diet, or vitamin B_1 *plus* lactoflavin (the latter in the form of crystalline lactoflavin, or as egg-white in doses of 3–5 ml. daily). It was found that the only factor of importance in this respect was the daily quantity of vitamin B_1 administered. If less than 3 pigeon day doses were given daily the appearance of pellagra-like dermatitis was often delayed. In a few cases, examples of which are shown in the accompanying curves (Fig. 5), spontaneous healing of the "pellagra" was observed while these subliminal doses of vitamin B_1 were being given, either with or without the simultaneous appearance of polyneuritic symptoms. But a subsequent increase in the dose of vitamin B_1 to 3–4 pigeon day doses daily brought out the pellagra-like dermatitis once more, at the same time suppressing the nervous symptoms. Thus it is an indispensable condition for the production of pellagra-like dermatitis that the animals be adequately supplied with vitamin B_1. Excessive doses of vitamin B_1, 6–8–10 pigeon day doses daily instead of 3–4, did not accelerate the development of the dermatitis, nor did they predispose towards its appearance. We are therefore unable to agree with Kellogg and Eddy [1933], who see a relationship between the development of pellagra-like dermatitis and a hypothetical toxic action on the part of vitamin B_1. It is also worthy of mention in this respect, that in our experiments excessive dosage with vitamin B_1 does not affect the weight curves of the animals. This must be taken as evidence on the one hand that the vitamin B_1 requirements of the animals were amply satisfied by 3–4 pigeon day doses, and on the other hand that the vitamin B_1 preparation employed was uniform in the biological sense.

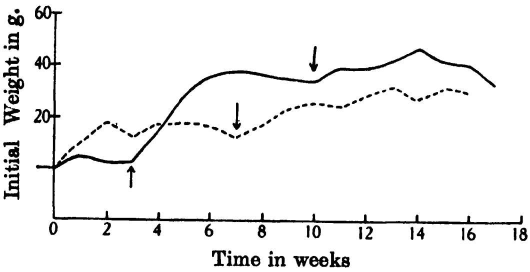

Fig. 5. Both animals received at first vitamin B_1 only (1·5 pigeon day doses daily); the animal —— from ↑ onwards, and the other rat - - - - - from the very commencement. One week before ↓ both rats showed definite signs of healing of the pellagra symptoms which had just recently appeared. Doubling of the dose of vitamin B_1 at ↓ produced an exacerbation of the pellagra symptoms.

We were unable to find any correlation between the season of the year when the experiments were performed, or between the initial weight of the experimental animals on the one hand and the development of "rat pellagra" on the other. The relation between the initial weight of the animals and the time taken to produce symptoms of "pellagra" is shown in the table below.

Initial body-weight (g.)	No. of exp. animals	First symptom of "pellagra" after av. no. of weeks	Animals ill twice	First symptom of "pellagra" of second illness after av. no. of weeks
26–35	21	7	—	—
36–45	51	7	1	13
46–55	35	7	—	—
56–65	17	6	2	8
66–75	21	7	5	7
76–85	17	9	7	10
86–95	18	9	14	9
96–105	16	6	13	6
106–115	7	7	6	7
116–125	4	7	3	8
126–135	4	6	4	6

The onset of the second illness in animals which have been successfully cured of a first attack of "pellagra" and are then re-submitted to the disease-producing conditions is determined by the intensity of the original therapeutic treatment. The reappearance of the disease may be considerably postponed by intensive treatment, which must be taken as evidence that the "pellagra-preventing factor" is capable of being stored in the body.

The skin manifestations met with when vitamin B_1 or B_1 *plus* lactoflavin (egg-white) are given may be distinguished readily and with certainty from those which appear when vitamin B_1 *plus* Peters's eluate are fed. The former symptoms are identical with the "pellagra-like" dermatitis of Goldberger and Lillie [1926], while the irregular skin manifestations of lactoflavin deficiency as seen with our experimental technique are chiefly of an unspecific nature.

The leading feature of "pellagra-like" dermatitis is the involvement of the most peripheral parts of the body. The disease begins with swelling and hyperaemia of the ears which is soon followed by the involvement of the fore and hind paws and the nasal region. The distribution is for the most part perfectly symmetrical but exceptions to this rule are met with occasionally. In the latter the symmetry is frequently, though not invariably, restored during the course of the illness. The second stage, which follows the swelling and hyperaemia, consists in the formation of crusts on the pinnae, which becoming thickened and indurated may develop into flower-like excrescences. Scabs form in the vicinity of the raw and inflamed nostrils, corresponding in their distribution to the follicular areas of the vibrissae, and also on the eyelids, which are gummed together in these cases. The vibrissae do not fall out. The paws undergo either a desquamation in small flakes or larger scales, or else their dorsal surfaces become markedly oedematous with a serous discharge, associated in many cases with gangrene of individual toes and secondary auto-amputation. Among the hairs on other parts of the body seborrhoeic scales or often only inspissated secretion are not infrequently found. The hair shows very little external alteration. In a few animals bleaching may be observed, hairs formerly black becoming grey. Ulceration of the buccal mucuous membrane, particularly the lower surface of the tongue, is a very common associated symptom. The pulse-rate per minute is normal (450–530); diarrhoea is a rare occurrence. If no specific treatment be undertaken the animals succumb, without exception, with progressive loss of weight which towards the end falls steeply.

The very striking differences in the characters of the skin manifestations afford the best proof that the disturbances of growth seen in lactoflavin deficiency on the one hand, and those met with during the administration of vitamin B_1, or B_1 *plus* lactoflavin (egg-white) on the other hand, do not depend on the same disturbances of metabolism. As we have already shown, administration of lactoflavin and of the "supplementary factor" in Peters's eluate both cause resumption of growth under suitable conditions. In the same way both lactoflavin and Peters's eluate bring about the disappearance of the skin manifestations in a short time.

The effect of lactoflavin in this respect runs parallel with the increase in growth and consists in disappearance of the fine whitish yellow scales and in shedding of the old hairs and their replacement by new ones of the normal density and gloss. This result is attained by 7–10 γ lactoflavin daily, whereas a manifold increase in the dose of Peters's eluate has no more effect on the skin changes than on growth, if the simultaneous administration of lactoflavin is withheld.

The healing of "pellagra-like" dermatitis under the influence of adequate doses of Peters's eluate begins with shedding of the vegetation on the ears, paws, nasal regions and eyelids. The ears when first freed from scabs are thickened and hyperaemic with ragged, irregular margins. In the vicinity of the eyes and nose, and in other parts of the body, the hair frequently comes out in tufts at the same time as the scabs and scales are shed, exposing bald areas until new hairs have reappeared. The transitory spectacle-like borders surrounding the eyes arise in this way. The last sign to disappear by which a case of healed "rat pellagra" may be recognised is the reddened, and later somewhat shiny, folded ears; finally, with sufficient treatment, even this residual symptom disappears.

In pellagra-like dermatitis developing during the administration of vitamin B_1 *plus* lactoflavin (10 γ daily), or vitamin B_1 *plus* egg-white (3–5 g. daily), administration of Peters's eluate in doses equivalent to 10 g. of fresh bakers' yeast will produce rapid healing of the skin changes with simultaneous increase in weight, equal to, or even exceeding, that demanded by the criterion (10 g. per week) (*cf. e.g.* Fig. 4). In very advanced cases the acceleration of growth in the first week of treatment may fall short of that anticipated, only to compensate for this delay in the 2–3 weeks following. The skin is generally restored to its normal condition after 3–4 weeks of treatment. Bleaching of the hairs, which may have arisen during the disease, generally persists after all the other symptoms have been successfully cured. With a dose of Peters's eluate equivalent to 5 g. of fresh bakers' yeast, the skin manifestations resolve in the same way, if somewhat more slowly, as with double this amount; but the increase in growth falls considerably short of the criterion. Still smaller doses leave even the skin manifestations unaffected. These subliminal doses may be of no avail even prophylactically. The symptoms however do not as a rule become intensified but exhibit considerable variations which in some instances may approach spontaneous healing. If the Peters's eluate be prepared from brewers' instead of from bakers' yeast, then doses equivalent to double the quantity of the original yeast will be necessary.

From the experiments described thus far, the fact emerges that the pellagra-preventing substance cannot be identical with lactoflavin but should be looked for in Peters's eluate. The correctness of this assumption is strongly confirmed by the following observations.

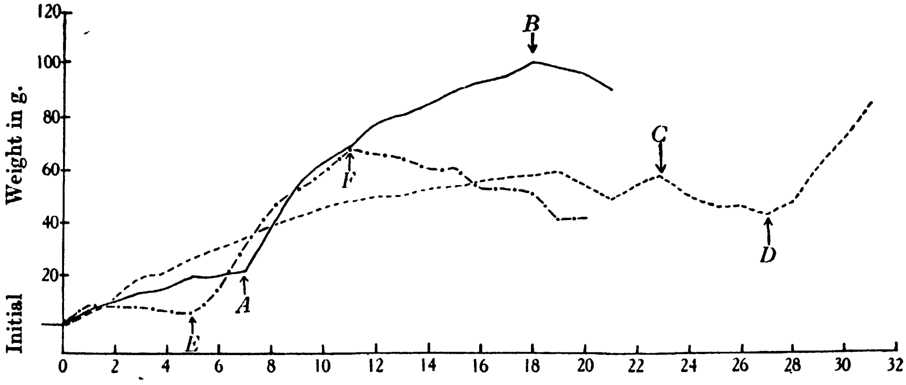

Fig. 6. The rat —— at first received vitamin B_1 + Peters's eluate only; at *A* lactoflavin was added, and at *B* Peters's eluate was omitted, *i.e.* administration of vitamin B_1 + lactoflavin only. Result: "pellagra". The rat ----- was treated at first with vitamin B_1 + lactoflavin and with doses of Peters's eluate insufficient to produce growth. At *C* this rat was given vitamin B_1 + lactoflavin only. Result: "pellagra" which was cured by Peters's eluate (*D*), this time in large doses. The rat —·— received at first vitamin B_1 + lactoflavin. Addition of Peters's eluate at *E* and again omitted at *F*. Result: "pellagra".

In animals which have suffered either from deficiency of lactoflavin or from "pellagra" and are being treated with vitamin B_1 *plus* lactoflavin *plus* "supplementary factor" (Peters's eluate), and no longer show any pathological skin changes, if then Peters's eluate be suddenly omitted, the specific skin manifestations of pellagra-like dermatitis will infallibly reappear sooner or later. A few examples from this extensive series of experiments are given in Fig. 6. On the other hand elimination of lactoflavin with continued administration of Peters's eluate does not lead to the development of "pellagra".

The conclusion is therefore irresistible that the "pellagra-preventing substance" is contained in the flavin-free, charcoal eluate of Peters, and that it is not identical with lactoflavin. When engaged in proving the growth-promoting properties of lactoflavin in our earlier experiments [György, Kuhn and Wagner-Jauregg, 1933; 1934] we made use of Peters's eluate as a "supplementary factor". We then expressed the opinion that the active principle to which the properties of Peters's eluate were due was vitamin B_4. In the specific vitamin B_4 deficiency disease, as described by Reader, nervous symptoms resembling polyneuritis are prominent, whereas in our experiment these symptoms were completely lacking, the picture being dominated by the pellagra-like dermatitis. Furthermore we succeeded in curing the pellagra-like dermatitis by using, instead of Peters's eluate, a yeast preparation (marmite) autoclaved for 4 hours at p_H 10 [György, 1934]. This finding cannot be reconciled with the fact that vitamin B_4 is highly sensitive to alkalis (Reader). In this respect we may refer to our earlier remarks (p. 748). In our preliminary communication, to avoid misunderstandings, we chose for the component of the vitamin B_2 complex, which was to be regarded as the pellagra-preventing substance, the provisional designation of vitamin B_6, leaving open the question of its identity with the Y-factor of Chick and Copping [1930], and with the pigeon-factor B_5. We propose to adhere to this designation for the time being and shall return to the problem of nomenclature later.

It follows from the experiments described above that no causal significance can be ascribed to lactoflavin, either in respect of the development or the cure of "pellagra-like" dermatitis. The appearance of "pellagra" was observed in 189 cases in which vitamin B_1 *plus* lactoflavin was given, but not a single case was seen when B_1 *plus* B_6 (Peters's eluate) only were given. Pellagra-like dermatitis developed in 22 animals which were given vitamin B_1 only, with no simultaneous administration of lactoflavin. In these cases the administration of vitamin B_6 alone, in the form of Peters's eluate (*cf.* Fig. 7), eventually brought about the cure of the pellagra-like skin manifestation, just as it did also in the case of those animals which developed "pellagra" when given vitamin B_1 *plus* lactoflavin. Nevertheless the two cases differ in two respects. There is, as might be expected, no resumption of growth in the absence of lactoflavin on the one hand, in spite of the administration of vitamin B_6, and on the other hand the skin changes take much longer to clear up than when B_6 is given in combination with lactoflavin; although the "pellagra" shows considerable improvement after 4 weeks, it is by no means healed. The symptoms of lactoflavin-deficiency, where this exists, remain in all their intensity. It should however be pointed out that with more prolonged treatment all the manifestations of pellagra will eventually disappear under the influence of vitamin B_6 (Peters's eluate) alone. In many cases

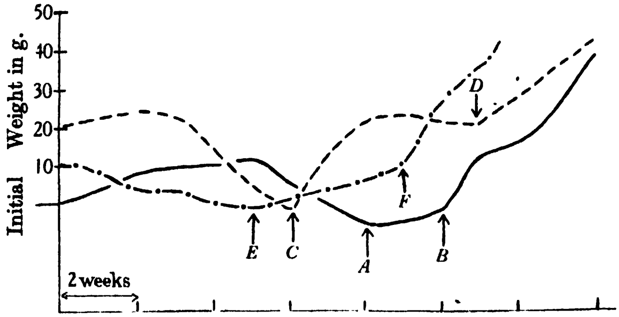

Fig. 7. The weight-curves of three rats which had developed "pellagra" with vitamin B_1 as the only supplement. At *A*, *C* and *E* addition of Peters's eluate, and at *B*, *D* and *F* of lactoflavin also.

where marked loss of weight has occurred before the beginning of treatment, administration of vitamin B_6 even without simultaneous administration of lactoflavin may cause a transitory stimulation of growth, but this increase comes to a stop after 1–2 weeks and can only be renewed by giving lactoflavin (Fig. 7). If feeding with lactoflavin is begun during a stage when the administration of vitamin B_6 has not yet completely cured the pellagra, the rapid improvement which sets in may be erroneously ascribed to the action of lactoflavin. The falsity of this conclusion has been finally proved by our experiments.

DISCUSSION.

Analysis of the vitamin B_2 complex into lactoflavin and a residual factor (or factors) constitutes the first step towards the successful analysis of this group of water-soluble vitamins. The growth-promoting action of lactoflavin and the presence of a lactoflavin-free "supplementary factor" which was necessary in order to demonstrate this growth-promoting action (György, Kuhn and Wagner-Jauregg) have been confirmed once more by the experiments described in this paper, in which a simplified and easily comprehensible technique was employed. There can be no doubt of the correctness of these findings, as they have since been confirmed by other workers, such as Euler *et al.* [1933; 1934], Chick and Copping [1934] and others.

The biological activity of lactoflavin was also recognised by Booher [1933; 1934], who claims, in a recently published paper, that a lactoflavin concentrate prepared by her stimulated growth in rats maintained on the diet of Bourquin and Sherman without any supplementary factor being present. This claim however rests upon the assumption that the lactoflavin prepared by Booher is comparable with the pure lactoflavin which was employed by us. A glance at the doses of the two preparations required is sufficient to invalidate this assumption. The daily requirement of Booher's preparation is 600γ, that of pure lactoflavin 7–10γ. It seems certain that the lactoflavin concentrate of Booher represents a heterogeneous mixture, which contains among other things the supplementary factor which we found it necessary to add to lactoflavin in our experiments.

The recent synthesis by Kuhn and Weygand [1934] of a lactoflavin which was biologically active under the usual conditions (*i.e.* in the presence of Peters's eluate as a supplementary factor) represents the climax of the series of researches which had as their object the isolation of lactoflavin as one of the components of the vitamin B_2 group.

The vitamin character of lactoflavin was first established by the growth test exclusively, while the relationship of lactoflavin to pellagra-like dermatitis, which according to the original definition, represented the real specific symptom of vitamin B_2 deficiency, was at first obscure. In the first place the technique originally employed failed to produce "pellagra". This essential condition was only satisfied after the experimental technique was modified. The utilisation of a crystalline or highly purified preparation of vitamin B_1 instead of an alcoholic extract of wheat as the source of this vitamin together with a diet otherwise free from vitamin B_1 led invariably to the development of "pellagra". In our experience with over 200 animals, the average time elapsing before the appearance of the specific skin manifestations was 7 weeks. Using this experimental technique it was found that lactoflavin had nothing to do with either the prevention or the cure of "pellagra-like dermatitis". The actual pellagra-preventing substance is the supplementary factor, or at all events a part of it, together with which lactoflavin is able to reveal its growth-promoting powers. A deficiency of this factor, which we had provisionally termed vitamin B_6, leads invariably to the appearance of "pellagra", even in the presence of lactoflavin.

The identification of the pellagra-preventing substance as one of the components of Peters's eluate also makes it possible to understand certain apparent discrepancies between our experimental results and the views held by Chick and her collaborators. Roscoe [1933, 1] observed pellagra-like dermatitis in about 50 % only of their experimental animals, and after an average experimental period of 10 weeks. In a personal communication Dr Chick states that the skin and manifestations were not generally severe in degree. Chick and Copping [1934] found that the action of lactoflavin upon the skin manifestations was a variable one. In many cases the "skin lesions" improved during the course of treatment with lactoflavin, while proving refractory in others. These results, which at first sight appear contradictory and irreconcilable with our own, are readily explicable in the light of our present knowldege. Chick and her collaborators employed Peters's eluate as the source of vitamin B_1 and thus continually supplied their rats with small quantities of pellagra-preventing substance in addition. Very often these quantities fell short of the threshold dose, as they were equivalent to only 0·6 g. of dried yeast, instead of 10 g. fresh yeast (equivalent to about 2 g. dried yeast). Further, brewers' yeast used by Chick is poorer in pellagra-preventing substance than bakers' yeast. At the same time, doses smaller than the therapeutic unit are effective prophylactically. It can therefore be understood why under these circumstances the appearance of pellagra in the experiments of Chick and her collaborators was inconstant, and that the symptoms of pellagra never reached the greatest intensity or developed in a short space of time. The slight therapeutic affect of lactoflavin occasionally observed by Chick and Copping [1934] also becomes easy to understand, since in our own experiments we have seen that lactoflavin occasionally accelerates the action of the "pellagra-preventing substance" and is thus apparently concerned in the healing process. On the other hand, the actual healing power is the property of the pellagra-preventing substance which was administered to the animals with the Peters's eluate.

Chick and Copping also suggest the identity of the supplementary factor (pellagra-preventing substance or vitamin B_6) with the factor Y previously described by them [1930]. This possibility was discussed also in a preliminary communication [György, 1934]. In view of the fact that Chick and Copping [1930] did not postulate any connection between the factor Y and the skin affections developed in absence of vitamin B_2, and that they did not take into account its character as supplementary substance within the vitamin B_2 complex, we thought it best, in order to avoid misunderstanding, to give the pellagra-preventing substance an entirely new designation.

The ultimate nomenclature of the vitamin B_2 complex will be a matter for the competent authorities to decide. We would urge that the generic term vitamin B_2 for the whole group be retained, and the individual components distinguished by special names. This question can be considered as already settled in the case of lactoflavin in the same way as the term ascorbic acid has been justified for vitamin C. For the time being the "pellagra-preventing substance" may be referred to as such, or as vitamin B_6, until its isolation has been accomplished, when it may receive its final designation. Elvehjem and Koehn [1934] have recently brought forward the proposal that the pellagra-preventing substance be termed vitamin B_2, and that lactoflavin be renamed, as they find that it has no connection with pellagra in chickens. In our opinion this proposal, if only from the point of view of the antecedent literature, would increase the confusion.

The systematic investigation of the vitamin B_2 complex does not form an end in itself. The clinician will view this analysis as only the first step essential for the better understanding of the relationship between the individual representatives of the vitamin B_2 complex and the corresponding specific diseases in man. Now that the separation of the pellagra-preventing substance (vitamin B_6) from lactoflavin has been accomplished, the problem of the prevention of human pellagra will have to be subjected to a new experimental investigation. In this respect nutritive products will be considered according to their content of vitamin B_6 and not as hitherto, according to their total vitamin B_2 content. Our experiments [György, 1935, 1] have already led to the noteworthy discovery that fish, the great value of which for the treatment of pellagra in man was expressly pointed out by Goldberger, is among the natural products richest in vitamin B_6. Thus the value of fish, *e.g.* herring, salmon *etc.* as a foodstuff is supported by this experimental finding.

Further research must be devoted to a close examination of the vitamin B_6 content of cereals, especially of maize, if only in view of the criticism of Aykroyd [1930] which was fully justified in the previous state of our knowledge. However, our ultimate object is the testing of concentrates rich in vitamin B_6 on cases of human pellagra; and experiments in this direction are already being contemplated. It will be the results of these experiments which will finally confirm or disprove Goldberger's theory, which regards human pellagra and rat pellagra (as well as "black-tongue" in dogs) as identical in nature.

In the absence of personal clinical experience of human pellagra I must refrain from taking up any definite point of view with regard to the identity of the clinical pictures in man and in the rat. On the other hand, I am struck by the resemblance of pellagra in rats to a peculiar disease of infancy, known as "pink disease" or acrodynia, which has only been recognised in the last 20 years. It would be of great value if the results of animal experimentation were applied to human pathology in this instance, especially as successes with the feeding of liver have recently been claimed in many quarters. One important clinical difference between pink disease and rat-pellagra is the absence of an increased pulse-rate in the animals, whereas in the disease of infants it is one of the most constant symptoms.

Although there are indications as to the part which the "pellagra-preventing factor" may play in human pathology, we are completely in the dark in the case of lactoflavin. The symptoms of lactoflavin deficiency in animals are not sufficiently striking or specific. Important information in this respect will probably only be obtained by empirical means.

The relation between the vitamin B_2 complex and Castle's so-called extrinsic factor (which, in combination with the intrinsic factor of the stomach, is responsible for the production of an antipernicious anaemia substance) must be decided by experiments specially devoted to this problem. All we know from the literature at the moment is, that this extrinsic factor is present in meat and in yeast autolysate (marmite), that it is very thermostable and soluble in water and alcohol [Castle and Rhoads, 1932; Wills, 1931; 1933; 1934]. According to Miller and Rhoads [1934] the extrinsic factor is also present in egg-white. Since egg-white is known to be rich in lactoflavin, it might appear that the extrinsic factor will be more closely related to lactoflavin than to the other known components of vitamin B_2. It is interesting to compare foodstuffs according to their lactoflavin contents on the one hand and their anti-anaemic powers on the other. The latter was studied by Whipple and Robscheit-Robbins [1927; 1928; 1929; 1930], working with nutritional anaemia, sustained and regulated by repeated bleedings in dogs, who found that fish flesh and fish liver were inactive, and our experiments have shown these to be the animal products which are poorest in lactoflavin [György, 1935, 1]. On the other hand, ox liver possesses the greatest anti-anaemic powers and the greatest lactoflavin content. Veal has a more potent anti-anaemic action than beef and is also richer in lactoflavin. Even allowing for the fact that the salts (Fe, Cu) and the haemoglobin contents of the nutritive products tested by Whipple and Robscheit-Robbins were also concerned in the therapeutic effects on anaemia investigated by them, the parallelism which is revealed between the anti-anaemic values and the lactoflavin contents is still a striking one. On the other hand, it must be pointed out that no sign of anaemia could be seen in our rats fed on a lactoflavin-free diet. True, this negative result might also be explained by the fact that rats are not suitable for the study of anaemia in this way, and further, the continued effect of repeated haemorrhages was also absent in the case of our rats in contradistinction to Whipple and Robscheit-Robbins's dogs. This question too will only be finally decided by direct clinical experiments.

In recent years the development of cataract in rats fed on a diet free from vitamin B_2 has been frequently described [Day *et al.*, 1931; Langston *et al.*, 1933]. In an extensive series of experiments in which over 500 rats were involved, we never observed this phenomenon either with complete or partial deficiency of the vitamin B_2 complex or with separate deficiencies of lactoflavin or vitamin B_6.

SUMMARY.

1. "Rat pellagra" can be produced, almost without exception, by the use of a crystalline or highly purified preparation of vitamin B_1 (3–4 pigeon day doses daily) added to a vitamin B-free basal diet.

2. The addition of lactoflavin, whether in the form of the pure substance (7–10 γ daily) or as egg-white (3–5 ml. daily), does not affect this result. Lactoflavin therefore can have no connection with "pellagra-like dermatitis". These observations extend over 211 animals.

3. The "pellagra-preventing" substance corresponds to the "supplementary substance" which is needed to demonstrate the growth-promoting action of lactoflavin. The combined administration of lactoflavin and the supplementary substance leads to a prolonged and marked increase in growth in rats in receipt of vitamin B_1 as the only member of the total vitamin B complex. Neither lactoflavin nor the "supplementary substance" by itself possesses any growth-promoting action.

4. Peters's "charcoal eluate" from yeast represents a suitable source of "supplementary substance" free from lactoflavin.

5. In view of its causal rôle in the development of "rat pellagra" the supplementary substance is termed the "rat pellagra-preventing factor" or, without making any further assumption, vitamin B_6.

6. The relations between the vitamin B_2 complex and its individual components and disease in man are discussed.

7. It is proposed that the designation vitamin B_2 be retained for the total complex. Lactoflavin might be known as such, and the pellagra-preventing substance as such, or provisionally as vitamin B_6.

It is with great pleasure that I express my deep gratitude to Prof. J. Barcroft whose hospitality has made possible the continuation of the experiments recorded in this and the accompanying papers.

My special thanks are due to Dr L. J. Harris who generously placed at my disposal the resources of the Nutritional Laboratory.

REFERENCES.

Aykroyd (1930). *Biochem. J.* **24**, 1479.

Booher (1933). *J. Biol. Chem.* **102**, 39.

—— (1934). *J. Biol. Chem.* **107**, 591.

Bourquin and Sherman (1931). *J. Amer. Chem. Soc.* **53**, 3501.

Castle and Rhoads (1932). *J. Amer. Med. Assoc.* **99**, 166.

Chick and Copping (1930). *Biochem. J.* **24**, 1744.

—— —— (1934). *Chem. Ind.* **53**, 874.

—— and Roscoe (1927). *Biochem. J.* **21**, 698.

Day, Langston and O'Brien (1931). *Amer. J. Ophth.* **14**, 1005.

Elvehjem and Koehn (1934). *Nature*, **134**, 1007.

Euler and Karrer (1933). *Arkiv. Kemi.* **11 B**, 16.

—— —— Adler and Malmberg (1934). *Helv. Chim. Acta*, **17**, 1157.

Goldberger and Lillie (1926). *U.S. Pub. Health Rep.* **41**, 1025.

Gurin, Eddy, Denton and Ammerman (1931). *J. exp. Med.* **54**, 421.

György (1934). *Nature*, **133**, 498.

—— (1935, 1). *Biochem. J.* **29**, 760.

—— (1935, 2). *Biochem. J.* **29**, 767.

—— van Klaveren, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 236.

—— Kuhn and Wagner-Jauregg (1933, 1). *Naturwiss*, **21**, 560.

—— —— —— (1933, 2). *Klin. Woch.* **12**, 1241.

—— —— —— (1934, 1). *Z. physiol. Chem.* **223**, 21.

—— —— —— (1934, 2). *Z. physiol. Chem.* **223**, 27.

—— —— —— (1934, 3). *Z. physiol. Chem.* **223**, 241.

Hogan and Richardson (1932, 1). *Mo. Agric. Exp. Sta. Research Bull.* **178**, 3.

—— —— (1932, 2). *J. Biol. Chem.* **97**, vii.

—— —— (1933). *J. Biol. Chem.* **100**, lv.

Kellogg and Eddy (1933). *Science*, **33**, 609.

Kinhersley, O'Brien, Peters and Reader (1933). *Biochem. J.* **27**, 225.

Kuhn, György and Wagner-Jauregg (1933, 1). *Ber. deutsch. chem. Ges.* **66**, 317.

—— —— —— (1933, 2). *Ber. deutsch. chem. Ges.* **66**, 576.

—— —— —— (1933, 3). *Ber. deutsch. chem. Ges.* **66**, 1034.

—— and Weygand (1934). *Ber. deutsch. chem. Ges.* **67**, 2084.

Langston, Day and Cosgrove (1933). *Arch. Ophth.* **10**, 508.

Medical Res. Council (1932). *Spec. Rep. Ser.* No. 167.

Miller and Rhoads (1934). *New. Eng. J. Med.* **211**, 921.

Reader (1929). *Biochem. J.* **23**, 689.

—— (1930, 1). *Biochem. J.* **24**, 77.

—— (1930, 2). *Biochem. J.* **24**, 1827.

Robscheit-Robbins (1929). *Physiol. Rev.* **9**, 666.

Roscoe (1933, 1). *Biochem. J.* **27**, 1533.

—— (1933, 2). *Biochem. J.* **27**, 1537.

Sherman and Sandels (1929). *Proc. Soc. Exp. Biol. Med.* **18**, 193.

—— —— (1931). *J. Nutrition*, **3**, 395.

Sure, Kik and Smith (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 498.

Thatcher, Sure and Walker (1931). *Arch. Path.* **11**, 425.

Warburg and Christian (1932, 1). *Biochem. Z.* **254**, 438.

—— —— (1932, 2). *Naturwiss.* **20**, 980.

—— —— (1933). *Biochem. Z.* **266**, 377.

Whipple (1928). *J. Amer. Med. Assoc.* **91**, 863.

—— and Robscheit-Robbins (1927). *Amer. J. Physiol.* **79**, 260.

—— —— (1930). *Amer. J. Physiol.* **92**, 388.

Wills (1931). *Brit. Med. J.* 1059.

—— (1933). *Lancet*, 1283.

—— (1934). *Ind. J. Med. Res.* **21**, 669.

Windaus, Tschesche, Ruhkopf, Laquer and Schultz (1931). *Nachr. Ges. Wiss. Göttingen*, **3**, 207.

LXXXVII. INVESTIGATIONS ON THE VITAMIN B_2 COMPLEX.

II. THE DISTRIBUTION OF LACTOFLAVIN AND OF THE "PELLAGRA-PREVENTING FACTOR" (VITAMIN B_6) IN NATURAL PRODUCTS OF ANIMAL ORIGIN.

By PAUL GYÖRGY.

From the Physiological and Nutritional Laboratories, Cambridge.

(*Received January 31st, 1935.*)

THE isolation of lactoflavin as one of the components of the vitamin B_2 complex and the separation of the actual "pellagra-preventing factor"—provisionally termed vitamin B_6 [György, 1934; 1935]—made it possible to test natural products for their content of lactoflavin and vitamin B_6 by biological experiments. All the statements in the literature regarding the natural distribution of vitamin B_2 need re-examining, since the complex nature of vitamin B_2 was not formerly realised. This criticism applies to our own earlier communication [György *et al.*, 1934, 1] in which the quantitative data concerned only the total vitamin B_2 complex and not its individual components.

The example of egg-white illustrates the gross errors to which disregard of this complexity may lead. Egg-white is commonly regarded as a natural product rich in vitamin B_2 [Chick and Roscoe, 1929]. As we have pointed out in the preceding paper, this assertion holds good only for lactoflavin, and not for the "pellagra-preventing substance". As source of lactoflavin 3 ml. of fresh egg-white provide 1 rat day dose, whilst the vitamin B_6 content is too small to be demonstrable by biological methods.

Miss Chick has kindly pointed out to me that she always used egg-white in the cooked or dried state. Since the possibility had to be considered that the "pellagra-preventing substance" might be more easily absorbed from boiled than from unboiled egg-white, we repeated the experiments with boiled egg-white. However, we were unable to produce the slightest therapeutic effect in eight animals suffering from "pellagra", even with 3–10 g. of boiled egg-white; three animals died during treatment and the others could only be saved and eventually cured by the administration of active preparations.

Using the experimental technique described below, we determined the lactoflavin and vitamin B_6 contents of various natural products of animal origin. In the case of lactoflavin the titre was determined by means of the growth test, the skin symptoms being too irregular and frequently insufficiently obvious; the titre of vitamin B_6 was determined by its effect on the skin changes. In estimating the lactoflavin values, crystalline (or highly purified) vitamin B_1 (3–4 pigeon units), and B_6, in the form of Peters's eluate from bakers' yeast (equivalent to 10 g. of fresh bakers' yeast), were added to the diet. The additions to the diet for the elimination of vitamin B_6 were again vitamin B_1 and, in this instance, pure lactoflavin (10 γ daily) or egg-white (3–5 ml. daily).

Later on lactoflavin was generously placed at my disposal by my colleagues Kuhn and Wagner-Jauregg, while vitamin B_1 and Peters's eluate were supplied through the kindness of the I.G. Farbenindustrie Wuppertal-Elberfeld (Germany). The Peters's eluate used displayed a constant vitamin B_6 value, so that the experimental results obtained were strictly comparable. The Peters's eluate originally employed was prepared by myself.

A large part of this work was devoted to investigations of muscle tissue of various origins, since we had previously found [György *et al.*, 1934, 1] striking differences in the total contents of vitamin B_2 complex in red and white poultry muscle, and in heart-muscle, veal and beef. In the chicken, dark muscle was found to be richer in total vitamin B_2 than white muscle. On the other hand, these earlier experiments with the diet of Bourquin and Sherman, showed that ox heart muscle possessed the highest content of vitamin B_2 and skeletal muscle the lowest, the value for veal being intermediate between the two.

We repeated these latter experiments, using the new method in which the animals received, to begin with, only vitamin B_1 and the meat concerned in addition to the basal diet. After an initial increase, which, with the meat supplements, continued for 6–8 weeks, the weight became stationary. At this stage of the experiment a third supplement was added, one group receiving lactoflavin and the other vitamin B_6 in the form of Peters's eluate, whereupon a fresh increase in weight set in. We obtained the following mean weekly figures (in g.) for the two periods (*a*) with vitamin B_1 alone, (*b*) vitamin B_1 *plus* lactoflavin or vitamin B_6:

		Weekly increase in weight in g.	
Meat fed	Daily amount (g.)	(*a*) With vit. B_1	(*b*) After additional administration of lactoflavin
Heart muscle	0·75	6	3
Veal	2	8	7
Beef	2	3	4
		(*a*) With vit. B_1	(*b*) After the addition of vit. B_6
Heart muscle	0·75	8	11
Veal	2	10	8
Beef	3	6	12

Our earlier conclusions were thus fully supported by these preliminary experiments, which also yielded fresh information. In order to produce the standard rate of growth, heart muscle, at all events in the doses given, required the addition of vitamin B_6 rather than of lactoflavin. At first sight the same conclusion appeared to hold good for beef also, but the employment of different quantities of meat (2 and 3 g. respectively) in the two groups did not allow a definite comparison to be made.

Clearer results were obtained in experiments in which two groups of animals were given lactoflavin and Peters's eluate respectively, together with vitamin B_1 from the commencement, before supplementary feeding with meat was begun.

As determined by the growth test 0·75 g. heart muscle, 2 g. veal or 3 g. beef contain 1 rat day dose of lactoflavin. The results are thus of the same order as those obtained previously with the experimental methods which have since been superseded. There were no marked differences between the three kinds of meat in respect of their contents of vitamin B_6.

In their curative effects upon "pellagra-like" dermatitis 0·75 g. heart muscle, 0·6–0·75 g. of veal or 1 g. beef are of equal value.

The values for lactoflavin are much higher in veal, and even more so in beef, than the values for vitamin B_6. For these meats, therefore, lactoflavin represents the limiting factor. In this respect, striated skeletal muscle differs widely from egg-white, which as we have already seen, is practically free from vitamin B_6 and comparatively rich in lactoflavin.

		With vits. B_1+B_6 weekly increase in weight in g.	With vit. B_1 + lactoflavin	
Meat fed	Daily amount (g.)		Weekly increase in weight in g.	Healing of pellagra-like dermatitis
Heart muscle	1	12	9	Yes
	0·75	11	6	Yes
	0·5	6	3	No
Veal	3	12	12	Yes
	2	10	9	Yes
	1	—	7	Yes
	0·75	1	6	Yes
	0·5	—	5	Inconstant
Beef	3	12	9	Yes
	2	5	8	Yes
	1·5	5	7	Yes
	1	—	5	Yes
	0·75	—	5	No
	0·5	—	3	No

Fish muscle lies right at the other end of the series; salmon (fresh and frozen), herring and haddock were investigated.

		With vits. B_1+B_6 weekly increase in weight in g.	With vit. B_1 + lactoflavin	
Fish muscle fed	Daily amount (g.)		Weekly increase in weight in g.	Healing of pellagra-like dermatitis
Fresh salmon	3	0	—	—
	2	2	—	—
	1	1	8	Yes
	0·75	—	9	Yes
	0·5	—	8	Yes
Frozen salmon	1	—	8	Yes
	0·5	—	7	Yes
Herring	3	6	—	—
	2	4	10	Yes
	1	2	10	Yes
	0·5	—	6	Yes
Haddock	3	2	—	—
	2	1	9	Yes
	1	1	—	—
	0·75	—	9	Yes
	0·5	—	3	Yes

It will be seen that fish muscle is inadequate as a source of lactoflavin even in doses of 3 g. daily; only in the case of herring is there a slight increase of growth and even this, despite the high dosage, falls far short of the standard amount. In view of these results, the lactoflavin content of fish muscle must be extraordinarily small. In contrast to this however fish muscle is remarkable for its richness in vitamin B_6. The therapeutic dose of all the varieties of fish investigated was in the region of 0·5 g. daily, or even less. Fish muscle is the only natural product which we have so far investigated, which is practically free from lactoflavin, and is at the same time rich in vitamin B_6. In the absence of artificial preparations of lactoflavin and of vitamin B_6, egg-white and fish muscle may therefore be used as sources of lactoflavin and vitamin B_6 respectively in biological experiments. Frozen fish contains the same quantity of "pellagra-preventing substance" as fresh fish.

Not even the highest doses of fish muscle were able to exercise any favourable influence on the skin manifestations met with in cases of deficiency of lactoflavin [György, 1935]. The animals were refractory both as regards growth and the healing of the skin manifestations. The addition of lactoflavin to only 1 g. of fish muscle gave rise to a marked increase in weight which continued for weeks with undiminished intensity (Fig. 1). The impression was gained that the requirement of lactoflavin was lower in the presence of Peters's eluate + fish muscle than in experiments with Peters's eluate alone.

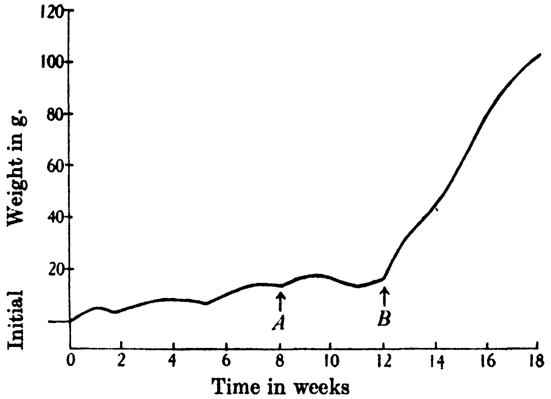

Fig. 1. Salmon in doses of 1 g. (beginning at *A*) is unable to supplement Peters's eluate + vitamin B_1. Lactoflavin (10γ daily) added at *B*.

By means of separate estimations of lactoflavin and vitamin B_6 we were able to confirm our previous conclusions [György *et al.*, 1934, 1], regarding the greater biological value of the red muscles of the leg of chickens as compared with the white muscles of the breast.

Meat fed	Daily amount (g.)	With vits. $B_1 + B_6$ weekly increase in weight in g.	With vit. B_1 + lactoflavin: Weekly increase in weight in g.	With vit. B_1 + lactoflavin: Healing of pellagra-like dermatitis
Leg of chicken	3	11	13	Yes
	2	6	—	—
	1	4	8	Yes
Breast of chicken	3	8	12	Yes
	2	3	—	—
	1	2	4	Yes

As in the case of striated mammalian muscle (beef and veal), poultry meat contains relatively more "pellagra-preventing" substance than lactoflavin. In the latter case however the difference does not amount to much, especially if the results of the growth tests are compared. Healing of pellagra-like dermatitis is obtained with red muscle of the leg as well as with white breast meat, smaller doses being required for this purpose than are necessary to produce the standard growth in animals fed with lactoflavin-free supplements, but in receipt of Peters's eluate. The red muscle is richer than the white, in lactoflavin especially, but also, according to the growth test, in vitamin B_6.

The contents of lactoflavin and of pellagra-preventing substance (vitamin B_6) of the smooth muscle of the chicken's gizzard are lower than those of striated muscle including the white muscle of poultry. The "rat day dose" of lactoflavin is equivalent to 3–4 g., and the antidermatitic, therapeutic, "rat day dose" of vitamin B_6 is equivalent to 2 g. of gizzard muscle. Daily doses less than 2 g. are unable to bring about healing of the pellagra-like dermatitis.

Among the viscera, mammalian liver (beef) was examined for its contents of lactoflavin and vitamin B_6. Liver is the organ which is richest in vitamin B_6 and lactoflavin. The "rat day dose" for lactoflavin was found to be present in 0·2–0·3 g., and for the "pellagra-preventing" factor in 0·3 g. daily. Thus the liver, like the heart and unlike striated and smooth muscle, appears to contain more lactoflavin than vitamin B_6.

Compared with mammalian liver, fish liver is very poor both in lactoflavin and in pellagra-preventing substance. The small lactoflavin content of fish liver had previously been established by means of a chemical, or fluoroscopic, method by Kuhn *et al.* [1934], and also by Euler and Adler [1934, 1, 2]. These authors agree that the lactoflavin content of cod liver is 0·5γ per g. fresh weight (in mammalian liver 15·9γ). According to the present biological investigations the lactoflavin "rat day dose" of cod-liver is over 3 g. per day. Such biological tests are preferable to chemical analysis, among other reasons because there is the possibility that mixtures of active and inactive flavins are present in all natural products, between which purely chemical analysis is, at present, unable to distinguish. The average weekly gain in weight with this dose of 3 g. amounted to 6 g. In contrast, bovine liver in doses 1/10 of these, *i.e.* 0·3 g. daily, caused an average increase in weight of 12 g. weekly. This comparison constitutes the most impressive proof of the poverty of fish liver in lactoflavin. The smallest therapeutic dose which will cure dermatitis is about 2 g. of cod liver daily, with which dose the effect on the weight curve is just noticeable.

Cow's milk contains about the same amount of lactoflavin as of vitamin B_6, the "rat day dose" for both components of the vitamin B_2 complex being equivalent to about 10 ml. These investigations were performed in the summer months.

Daily dose of milk (ml.)	With vits. B_1+B_6 weekly increase in weight in g.	With vit. B_1 + lactoflavin	
		Weekly increase in weight in g.	Healing of pellagra-like dermatitis
15	—	14	Yes
10	12	13	Yes
5	4	5	Mostly not

If, in unsuccessful treatments with 5 ml. cow's milk, Peters's eluate be substituted, a marked increase in weight sets in which exceeds the increase which usually occurs with this dose of Peters's eluate (Fig. 2). The preliminary feeding of milk therefore appears to reinforce the combined actions of lactoflavin and vitamin B_6 (Peters's eluate) on growth, much in the same way as was observed with fish muscle.

The investigations of Warburg and Christian [1932; 1933] have shown that in the oxidation of hexosemonophosphoric acid by the "yellow oxidation-ferment" (Warburg), a co-ferment from horse's red blood corpuscles plays an important rôle. After the identity of the coloured component of this flavoprotein [György *et al*, 1934, 2] with lactoflavin had been established, it became of importance to submit horse's red blood corpuscles to biological examination to test their possible activity in the second missing component, that is, the pellagra-preventing substance vitamin B_6. However, 2 ml. of an emulsion of erythrocytes obtained by centrifuging freshly drawn horse blood were shown to be free from the missing supplementary factor (vitamin B_6). In addition to this the content of lactoflavin was also very low. In the same way, we were unable to obtain either the slightest lactoflavin effect or pellagra-preventing effect with 2 ml. of horse serum. We were also unable to find any biologically active lactoflavin in the lens of the eye, even on administering as much as 8 g. of tissue daily.

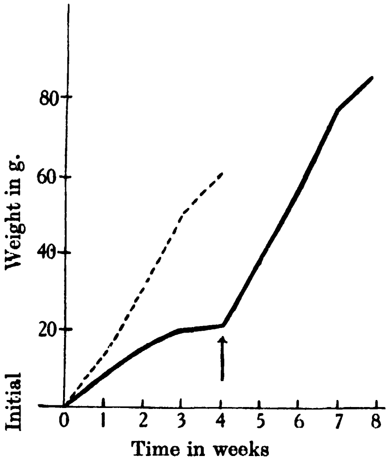

Fig. 2. 5 ml. milk do not contain sufficient pellagra-preventing substance to supplement vitamin B and lactoflavin (——). At ↑ Peters's eluate (equivalent to 10 g. fresh bakers' yeast) is substituted for the milk. The other weight curve (-----) shows the effect of 10 ml. milk in supplementing vitamin B_1 + lactoflavin.

The numerical results of these investigations on the contents of lactoflavin and vitamin B_6 of various natural animal products may be summarised in the two tables following:

	1 "rat day dose" of lactoflavin is contained in g.
Liver (beef)	0·2
Beef heart muscle	0·75
Veal	2
Beef muscle .	3
Leg of chicken	3
Breast of chicken	3–4
Chicken's gizzard	3–4
Egg-white	3
Milk	10 ml.
Horse serum	>2
Horse erythrocytes	>2
Fish liver (cod)	>3
Salmon muscle	>3
Herring ,,	>3
Haddock ,,	>3
Lens of eye	>8

	1 "rat day dose" of vit. B_6 is contained in g.
Liver (beef)	0·3
Salmon	0·5
Herring	0·5
Haddock	0·5
Veal	0·75
Heart muscle (beef)	0·75
Beef muscle	1·0
Leg of chicken	1·0
Breast of chicken	1·0
Chicken's gizzard	2·0
Fish liver (cod)	2·0
Milk	10·0 ml.
Horse serum	>2·0
Horse erythrocytes	>2·0
Egg-white	>10·0

While the lactoflavin values accepted in the above table are based on the results of the growth test, the titre values for the "pellagra-preventing substance" (vitamin B_6) are based on the antidermatitic therapeutic effects of the products examined for "rat pellagra". The values for lactoflavin and vitamin B_6 thus obtained are not therefore, strictly speaking, absolutely comparable and they hold good only for the method employed. However, these figures may be regarded as relatively correct.

SUMMARY.

Results are given of quantitative estimations of the vitamin B_6 and lactoflavin activities of foodstuffs of animal origin. It has been shown that various products differ in their absolute and relative contents of lactoflavin and vitamin B_6. Fish muscle (herring, salmon and haddock) is a rich source of the "antipellagra" factor but is practically devoid of lactoflavin. On the other hand, egg-white despite its high content of lactoflavin has no antipellagra activity.

Further, results are given for the lactoflavin and vitamin B_6 contents of the striated muscles of ox, calf and chicken, the heart muscle of ox, chicken gizzard, liver and milk.

REFERENCES.

Chick and Roscoe (1929). *Biochem. J.* **23**, 498.
Euler and Adler (1934, 1). *Z. physiol. Chem.* **223**, 1.
—— —— (1934, 2). *Sv. Vet. Akad. Arkiv. Kemi.* **11 B**, No. 28.
György (1934). *Nature*, **133**, 498.
—— (1935). *Biochem. J.* **29**, 741.
—— Kuhn and Wagner-Jauregg (1934, 1). *Z. physiol. Chem.* **223**, 21.
—— —— —— (1934, 2). *Ber. deutsch. chem. Ges.* **66**, 1034.
Kuhn, Wagner-Jauregg and Kaltschmitt (1934). *Ber. deutsch. chem. Ges.* **67**, 1952.
Warburg and Christian (1932). *Biochem. Z.* **254**, 438.
—— —— (1933). *Biochem. Z.* **266**, 377.

LXXXVIII. INVESTIGATIONS ON THE VITAMIN B_2 COMPLEX.

III. THE INACTIVATION OF LACTOFLAVIN AND VITAMIN B_6 BY VISIBLE LIGHT.

BY PAUL GYÖRGY.

From the Physiological and Nutritional Laboratories, Cambridge.

(*Received January 31st, 1935.*)

THE inactivation of preparations containing vitamin B_2 by irradiation with visible light [György *et al.*, 1933, 1, 2] necessitated an extension of our experiments, for whilst the destruction of the activities of vitamins by ultra-violet light has been the subject of close study for some time, and has been successfully applied also to the study of vitamin B_2 [Hogan and Hunter, 1928], investigations by the present author in association with Kuhn and Wagner-Jauregg were the first to show that visible light can also exercise the same effect. The inactivation of lactoflavin by visible light is the logical consequence of what has later been shown to be its nature as a dyestuff.

Our first observations were concerned mainly with relatively unpurified concentrates of lactoflavin, which for the most part still contained considerable amounts of "vitamin B_6". In order to demonstrate the effect of light, we mostly employed an artificial source of light (500 K.W. bulb). The complete loss of the vitamin B_2 activity of liver concentrate, not only after direct irradiation but also after it had been exposed in a room to diffuse daylight for 18 months, has previously been reported [György *et al.*, 1934, 1]. It naturally seemed important to correlate this photosensitivity of lactoflavin with the conditions of occurrence of pellagra in man; but after it was seen that lactoflavin was not identical with the pellagra-preventing substance, and that in consequence deficiency of the former could play no part in the development of pellagra, any such correlation lost a great deal of its practical significance. Evidence was still lacking on the photosensitivity of the newly discovered factor, the actual "pellagra-preventing factor", vitamin B_6. This gap can be filled now that the production of pellagra-like dermatitis no longer presents any technical difficulties.

The first experiments of this nature served to confirm our earlier findings regarding the inactivation of lactoflavin by visible light. A concentrate from cow's liver prepared in the usual way [*cf.* György *et al.*, 1934, 2] was placed in a thick-walled, rectangular glass bottle at a distance of about 30 cm. from a 500 K.W. electric bulb, for 24 hours; cooling was carried out by a fan. Irradiation was performed not only at a neutral reaction, but in two further specimens at acid (p_H 2–3) and alkaline (p_H 9–10) reactions. At the termination of the irradiation the two latter solutions were neutralised, as was also another control solution which had not been irradiated but kept for 24 hours at the same alkaline reaction. This control was necessary because, as we have already shown [György *et al.*, 1934, 1] when confirming the finding of Chick and Roscoe [1929], the activity of a vitamin B_2 concentrate may diminish in intensity at alkaline reactions, even at room temperatures. Rats, which for the few weeks preceding had received the basal diet and supplements of vitamin B_1 (3 pigeon units daily), were treated with irradiated and non-irradiated specimens of the liver concentrate (L_2; 4 ml. daily, equivalent to 2 g. fresh liver). The animals were already stationary in growth but showed no signs as yet of pellagra-like skin changes owing to the short time the experiment had been in progress. After the administration of specimens of the liver concentrate, we obtained the following figures for growth (in g. weekly) in the first group:

Non-irradiated		Irradiated		
Neutral	Alkaline	Neutral	Acid	Alkaline
g.	g.	g.	g.	g.
9	9	1	3	2

A second group of animals reacted similarly. Growth, which failed to increase in the animals treated with the irradiated specimens of liver concentrate, could be restored once more by the addition of lactoflavin (10 γ daily). The exact data in Fig. 1 give an impressive proof of the inactivation of lactoflavin by visible light.

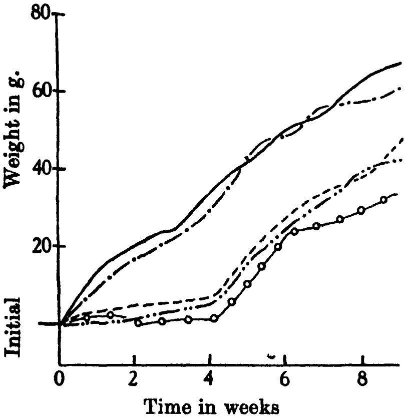

Fig. 1.

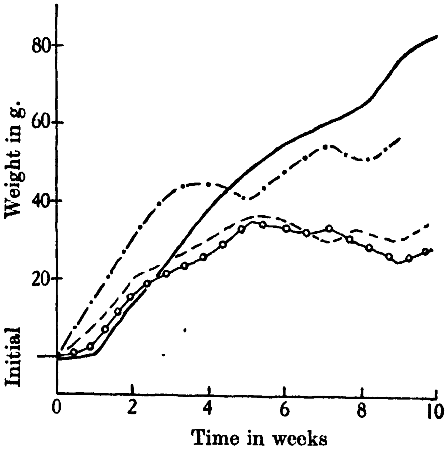

Fig. 2.

Fig. 1. The weight curves of five rats which were given vitamin B_1 + liver concentrate irradiated at neutral (o—o), at alkaline (—·—) and at acid (– – –) reactions: and non-irradiated liver concentrate at neutral (——) and at alkaline (—·—) reactions. Addition of lactoflavin at the end of the 4th week.

Fig. 2. Weight curves of rats which, apart from vitamin B_1 + lactoflavin, received Peters's eluate daily, the latter irradiated at neutral (—·—) or at alkaline (o—o) reactions, and non-irradiated at neutral (——) or at alkaline (– – –) reactions.

The inactivation of a concentrate of vitamin B_2 by irradiation with visible light is however not limited solely to the lactoflavin. Experiments carried out for a similar length of time with suitable doses of a second liver concentrate (L_3; 1·5 ml. = 1 g. of fresh liver) submitted to the same treatment, showed conclusively that vitamin B_6 was destroyed by visible light. In this experiment the specimens of liver concentrate were fed to animals in receipt of a basal diet supplemented by vitamin B_1 *plus* lactoflavin. In the following table are given the mean values of the weekly increases in weight over a period of 10 weeks and indications of the appearance or otherwise of symptoms of pellagra.

It will be seen from this table that, despite the addition of lactoflavin, the growth-promoting activity has been markedly diminished by irradiation with visible light. The differences in the weight curves do not appear so much in the first weeks of the experiment as later on, when a definite flattening of the curve makes its appearance in the case of the irradiated specimens. With the largest doses employed (3 ml. daily) this diminution in the growth-promoting property is manifested only by the specimen irradiated at alkaline reaction, and to some extent also by the non-irradiated specimen kept at alkaline reaction for 24 hours. Symptoms of pellagra did not develop. With doses of 2 and 1·5 ml. daily all the animals in receipt of the irradiated specimens developed definite symptoms of pellagra, whilst these were completely absent from the animals which were given corresponding non-irradiated control solutions. The slighter increases in weight of the animals treated with the non-irradiated alkaline liver concentrate as compared with those of the animals treated with the non-irradiated neutral liver concentrate are again very striking.

Liver concentrate daily ml.	Non-irradiated		Irradiated		
	Neutral g.	Alkaline g.	Neutral g.	Acid g.	Alkaline g.
3	12 No pellagra	9 No pellagra	13 No pellagra	10 No pellagra	7 No pellagra
2	12 No pellagra	7 No pellagra	7 Pellagra	9 Pellagra	8 Pellagra
1·5	8 No pellagra	6 No pellagra	3 Pellagra	6 Pellagra	3 Pellagra

Very similar results were obtained in further experiments with heart concentrate (1 ml. = 0·5 g. cow's heart). Observations extended over 10 weeks in this case also. The administration of heart concentrate to animals which again had been receiving vitamin B_1 *plus* lactoflavin from the commencement of the experiment, was begun quite early, before the appearance of pellagra-like symptoms. The latter then developed, if at all, during the period in which the heart concentrate was administered.

Heart concentrate daily ml.	Non-irradiated		Irradiated		
	Neutral g.	Alkaline g.	Neutral g.	Acid g.	Alkaline g.
5	9 No pellagra	7·5 No pellagra	4 Pellagra	6 Pellagra	6 Incipient pellagra

These and similar experiments, which, to avoid repetition, will not be given here, appear to prove that the pellagra-preventing substance is destroyed by visible light. The objection that the inactivation is an indirect effect of the sensitising action of the fluorescent lactoflavin, which is also present in concentrates of heart and liver, is disposed of, or if not completely disposed of at least rendered extremely improbable, by the fact that the fall in vitamin B_6 activity also occurs in the specimens irradiated at strongly acid or alkaline reactions. As is well known, lactoflavin no longer shows any fluorescence at these extreme reactions [Kuhn *et al.*, 1933]. Experiments which were performed with lactoflavin-free solutions of vitamin B_6, with Peters's eluate, and with concentrates or alcoholic extracts of salmon, also oppose the theory of the indirect nature of the photo-inactivation of vitamin B_6, at all events so far as lactoflavin as the photosensitive agent is concerned.

Fig. 2 gives the weight curves of four animals treated with Peters's eluate before and after irradiation (at neutral and alkaline reactions). Here again it will be seen that even irradiation at neutral reaction lowers the rate of growth in experiments extending over corresponding lengths of time. This effect is less marked with the specimen irradiated at alkaline reaction than with a non-irradiated control specimen which was kept at alkaline reaction. Both specimens gave rise to a much smaller increase in growth than did the original concentrate. There was however a marked difference in the development of rat-pellagra. Pellagra-like symptoms were only seen in animals which received the irradiated specimens of Peters's eluate, independently of whether irradiation were carried out at alkaline or neutral reaction.

Specimens of Peters's eluate (equivalent to 10 g. fresh baker's yeast) irradiated at alkaline reaction were compared with non-irradiated controls in two further experiments. The corresponding figures for growth (average per week) were

	Non-irradiated g.	Irradiated at alkaline reaction g.
1.	10	5
2.	12	2

Here again symptoms of pellagra appeared only in those animals which were given irradiated Peters's eluate. By doubling the dose in the second experiment we were able to bring about disappearance of the pellagra together with simultaneous increase in growth (9 g. weekly).

In all the experiments mentioned so far a powerful electric light (500 K.W.) was used for the destruction of the pellagra-preventing substance and lactoflavin by irradiation for 24 hours. If the results obtained by this technique were in all respects uniform, the photo-inactivation produced by exposure to diffuse daylight for correspondingly longer periods was still more impressive.

A liver concentrate (L_4), in a thick-walled transparent glass bottle, was kept in a room with diffuse daylight behind a window of opal glass. When this specimen was compared with a control specimen kept in the same room, but in a dark bottle wrapped in black paper, it showed a very marked fall in its activity in lactoflavin and vitamin B_6. Our earlier work, which, as has already been mentioned, was concerned with the total vitamin B_2 content of a liver concentrate which had been exposed to diffuse daylight for 18 months, is thus not only supported by these new findings but supplemented in another way. It was now shown that under the influence of diffuse daylight alone the vitamin B_6 as well as the lactoflavin titre of this liver concentrate had fallen to a very marked extent. In Fig. 3 are reproduced the weight curves of two animals, one of which received in addition to the basal diet 4 ml. daily of a liver concentrate kept in the dark and the other 4 ml. of the specimen exposed to light; apart from this both animals were given 10 γ lactoflavin *plus* vitamin B_1. The animal treated with the specimen exposed to light died in 5 weeks of pellagra, with weight completely stationary, whilst in the control animal the pellagra healed completely and the body weight increased by 97 g. Two animals which had also been submitted to the same treatment (basal diet *plus* vitamin B_1 *plus* lactoflavin), and were also already suffering from pellagra, were given 2 ml. of the specimen not exposed to light, and as a result showed an average weekly increase in weight of 17 and 16 g. respectively (68 and 65 g. respectively in 4 weeks), associated with rapid cure of the pellagra symptoms. The "exposed" specimen given in the same dose was again unable to supplement the lactoflavin. The body weights of two other animals, otherwise submitted to the same conditions, showed an average weekly fall of 2 and 1 g.

respectively, whilst the pellagra symptoms were aggravated. The irradiated liver concentrate also failed as a source of lactoflavin, *i.e.* as a supplement to Peters's eluate (vitamin B_6), not only in doses of 2 ml. but even 4 ml. daily. Fig. 4 shows the weight curve of one of these animals, which received 2 ml. of irradiated liver concentrate *plus* Peters's eluate (equivalent to 10 g. fresh baker's yeast) in addition to the basal diet. The sudden effect of the addition of lactoflavin supplement in this case affords the best proof of the assumption that lactoflavin was, in fact, absent from the "exposed" liver concentrate, at least in the doses given.

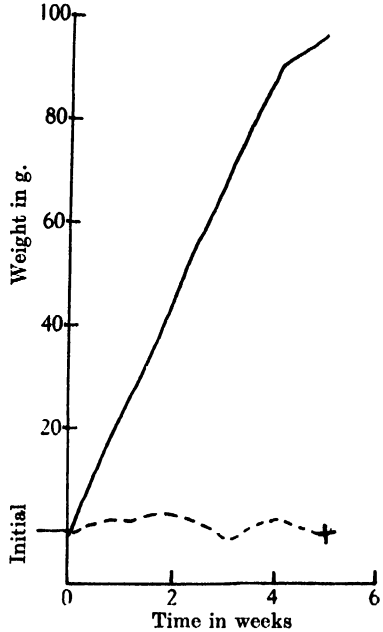

Fig. 3.

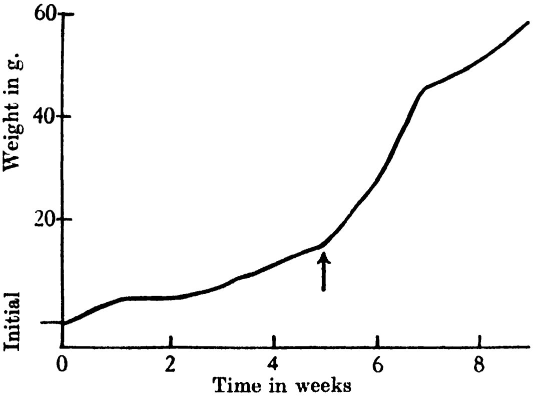

Fig. 4.

Fig. 3. Liver concentrate unexposed to light + vitamin B_1 + lactoflavin ——.
„ „ exposed „ „ „ - - - -.

Fig. 4. "Exposed" liver concentrate + vitamin B_1 + Peters's eluate. Addition of lactoflavin at ↑.

Experiments with the liver extract "campolon"[1] yielded results just as impressive as those already recorded. Specimens were exposed to diffuse daylight in thick-walled transparent bottles for 7½ weeks (Aug. 18th–Oct. 10th, 1934), (*a*) at a neutral reaction, (*b*) at a highly acid reaction with HCl (p_H 1–2), and (*c*) at a highly alkaline reaction with NaOH (p_H 11·0). Three corresponding controls were kept under similar conditions, except that they were protected from light of any kind. After 7½ weeks the specimens, neutralised and diluted 1:10, were tested biologically on animals which, apart from the basal diet, had received vitamin B_1 *plus* lactoflavin and already displayed symptoms of pellagra. The daily doses given were 5 ml. in one group, and 3 ml. in the second group, of each of the six different solutions of "campolon". In both series of experiments the non-irradiated specimens produced pronounced increase in weight with rapid healing of the pellagra. The non-irradiated specimens kept at alkaline reaction again gave rise to the slightest acceleration of growth only. Under the influence of diffuse daylight all the "campolon" solutions irrespective of their reactions

[1] Kindly supplied by the I.G. Farbendindustrie, Wuppertal-Elberfeld, Germany.

had completely lost their vitamin B_6 activity, at least in the doses in which they were given, 3–5 ml. daily. In spite of treatment, the symptoms of pellagra were aggravated without exception. A few animals died (Fig. 5). Substitution of the corresponding non-irradiated controls for the irradiated specimens resulted in the improvement anticipated, with increase in weight and resolution of the manifestations of pellagra (Fig. 6). As the animals were in receipt of vitamin B_1 *plus* lactoflavin, in addition to the "campolon" solutions, the difference in action between the "unexposed" and "exposed" solutions can only be explained by the differences in their contents of "pellagra-preventing substance" depending on its photo-inactivation.

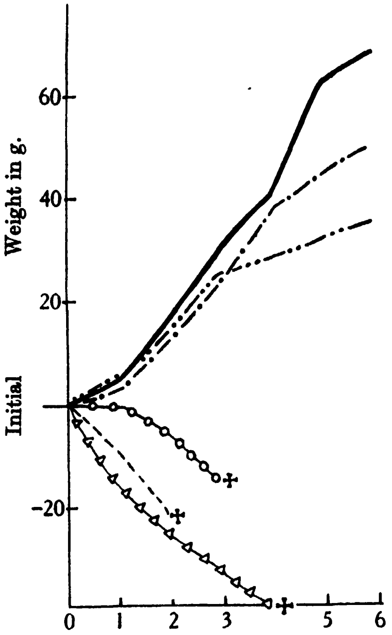

Fig. 5.

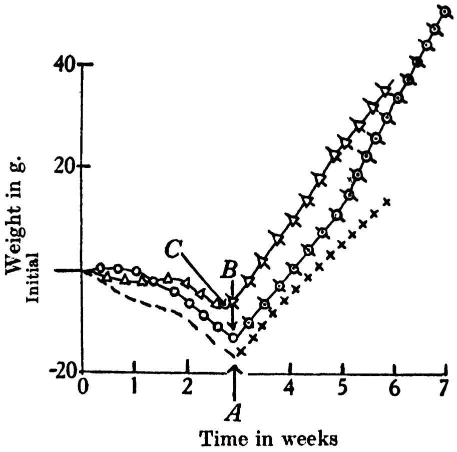

Fig. 6.

Fig. 5. Campolon solution "unexposed" at neutral reaction ——.
,, ,, ,, at acid ,, —·—.
,, ,, ,, at alkaline ,, —··—.
,, ,, "exposed" at neutral ,, – – –.
,, ,, ,, at acid ,, o—o.
,, ,, ,, at alkaline ,, △—△.
All the animals received vitamin B_1 and lactoflavin.

Fig. 6. (*a*) Campolon solution "exposed" at neutral reaction – – –. Exchanged at *A* for corresponding "unexposed" specimen.
(*b*) Campolon solution "exposed" at acid reaction o—o. Exchanged at *B* for corresponding "unexposed" specimen.
(*c*) Campolon solution "exposed" at alkaline reaction △—△. Exchanged at *C* for corresponding "unexposed" specimen.
All the animals received vitamin B_1 and lactoflavin.

The prolonged action of diffuse daylight, and especially of its visible portion, upon solutions which are practically free from lactoflavin, such as Peters's eluate, or concentrates or alcoholic extracts of salmon, leads to the destruction of the pellagra-preventing substance. For example, we obtained the following average figures for growth on using Peters's eluate exposed for 4 weeks to diffuse daylight.

	Non-irradiated			Irradiated		
	2 ml.	1·5 ml.	1 ml.	2 ml.	1·5 ml.	1 ml.
Weekly gain (g.)	10	8	7	4	1	0

All the animals had received lactoflavin from the very beginning and had displayed pellagra-like manifestations. Treatment with the unexposed specimens led, with all doses, to rapid resolution of the symptoms of pellagra. The highest dose of exposed specimen (2 ml. daily) also produced a definite improvement, if not a complete cure of the pellagra; but the pellagra was unaffected by 1·5 and 1 ml. daily.

The results of experiments with an ordinary concentrate and with an alcoholic extract of salmon used in addition to vitamin B_1 and lactoflavin were equally unequivocal. The alcoholic extract was prepared by the same process as that described by Bourquin and Sherman [1931] for the preparation of alcoholic extract of wheat. Both salmon extracts were exposed to the visible portion of the spectrum of daylight for 6 weeks (Aug. 29th–Oct. 11th, 1934). Control solutions were kept in the dark. Biological experiments performed later showed that the irradiated specimen had been completely inactivated (Figs. 7 and 8). This was in solutions which were, again, practically free from lactoflavin.

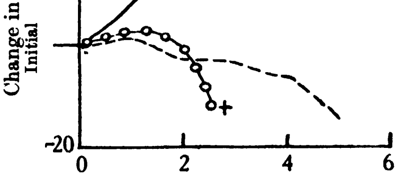

Fig. 7.

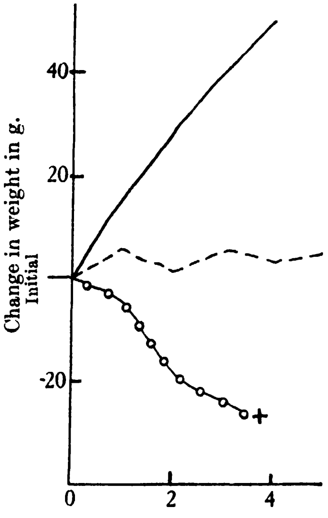

Fig. 8.

Fig. 7. Salmon concentrate, non-irradiated —— (3 ml.≡3·5 g. salmon), and "exposed" - - - (5 ml.), and likewise "exposed" o—o (3 ml.). All the animals received vitamin B_1 and lactoflavin.

Fig. 8. Alcoholic salmon extract, non-irradiated —— (3 ml.≡3 g. salmon), and "exposed" - - - (3 ml.), and likewise "exposed" o—o (5 ml.). All the animals received vitamin B_1 and lactoflavin.

All these identical results led to the indisputable conclusion that the pellagra-preventing substance (vitamin B_6) like lactoflavin is destroyed by visible light. This inactivation may also occur in the absence of lactoflavin. Under these circumstances it should be of importance to correlate this phenomenon, using the example of lactoflavin as an analogy, with the nature of the pellagra-preventing substance as a dyestuff, and not to regard it as a secondary photosensitisation. However the possibility must be considered that there may have been present, in all these solutions of vitamin B_6, yet another fluorescent substance, differing from lactoflavin, which might have exercised a photosensitising action.

Another point of interest in connection with the photo-inactivation of the pellagra-preventing substance is the fact that all the vitamin B_6 solutions examined so far have been coloured yellow, including the purified Peters's eluate, and that under the influence of diffuse daylight (from which the ultra-violet rays are excluded) they take on a darker, reddish brown colour. This change in colour is especially marked in concentrated solutions (3–5 rat day doses of vitamin B_6 in 1 ml.) of salmon extract, and also in Peters's eluate, *i.e.* again in the absence of lactoflavin. The curves in Fig. 9 give the quantitative data of the spectrophotometric examination of a salmon concentrate. (I am very much indebted to Prof. R. Kuhn for carrying out this investigation at his Institute.) The only fact which emerges from these measurements with certainty is the shift in the absorption of light of long wave-length in the irradiated extract, which was also obvious to the naked eye. The curves exhibited no characteristic maxima. Further chemical purification of the vitamin B_6 concentrates is now required; experiments in this direction have already been begun. The results of these experiments will decide whether this change of colour in solutions containing vitamin B_6 after exposure to the visible portions of diffuse daylight depends on the pellagra-preventing substance, or only on an associated substance which differs from it and is biologically inactive.

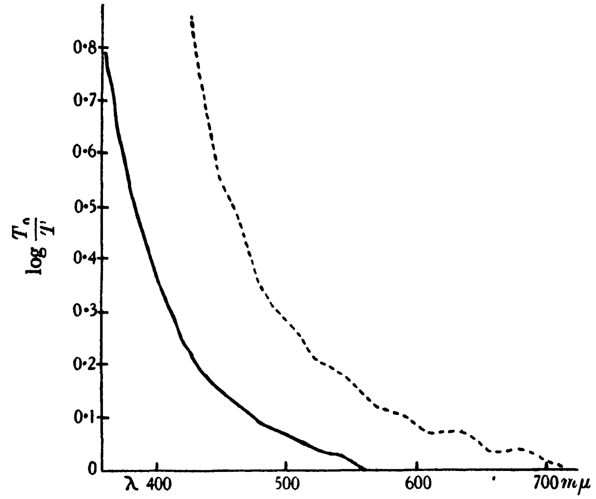

Fig. 9. Spectrogram of "unexposed" (——) and "exposed" (- - -) salmon concentrate (*cf.* Fig. 7) after concentration to 1:10.

According to Hogan and Richardson [1932, 1, 2; 1933] extracts of yeast or liver lose their activity as sources of pellagra-preventing substance on irradiation with ultra-violet light, and such loss of activity is much more rapid than is the case with vitamin B_1 activity. These authors have shown that this observation may be utilised in a process for the production of pellagra-like dermatitis in rats. This statement has been confirmed by the present writer in a large series of experiments involving over 80 rats. Daily doses of 0·1 g. of dry yeast which had been exposed in thin layers to ultra-violet radiation, led in fact almost constantly to the development of "rat pellagra", when given to rats fed on the vitamin B-free diet. However, this procedure possesses several disadvantages, which led us to discard its further use. In the first place numerous spontaneous healings were observed, and secondly there was a long "preparatory period" before the pellagra-like symptoms disappeared. Nevertheless, the use of this method on a large number of animals suffering from pellagra gave convincing evidence of the complete therapeutic inefficacy of lactoflavin, and of the curative effect of the "supplementary factor" (vitamin B_6).

The slight diminution in the growth-promoting action of solutions which have not been irradiated but which have been kept for some time at an alkaline reaction appears from the above experiment to be independent of any action of lactoflavin and probably of the pellagra-preventing substance also. This finding constitutes the sole support which we possess at present for the assumption that, in addition to the pellagra-preventing substance and vitamin B_1, Peters's eluate must contain yet another substance with the character of a vitamin. Further investigations are necessary to elucidate this last problem.

SUMMARY.

Both lactoflavin and the pellagra-preventing substance (vitamin B_6) may be destroyed by visible light. The inactivation of the pellagra-preventing substance also occurs in lactoflavin-free solutions, so that if it is dependent on an indirect photosensitisation it must be through some agency distinct from lactoflavin. The possibility is discussed and left open as to whether the pellagra-preventing substance is, like lactoflavin, a natural dyestuff.

REFERENCES.

Bourquin and Sherman (1931). *J. Amer. Chem. Soc.* **53**, 3501.
Chick and Roscoe (1929). *Biochem. J.* **23**, 498.
György, Kuhn and Wagner-Jauregg (1933, 1). *Naturwiss.* **21**, 560.
—— —— —— (1933, 2). *Klin. Woch.* **12**, 1241.
—— —— —— (1934, 1). *Z. physiol. Chem.* **223**, 21.
—— —— —— (1934, 2). *Z. physiol. Chem.* **223**, 241.
Hogan and Hunter (1928). *J. Biol. Chem.* **78**, 433.
—— and Richardson (1932, 1). *Mo. Agric. Exp. Sta. Research Bull.* **178**, 3.
—— —— (1932, 2). *J. Biol. Chem.* **97**, vii.
—— —— (1933). *J. Biol. Chem.* **100**, lv.
Kuhn, György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 317.

LXXXIX. FLAVIN AND THE PELLAGRA-PREVENTING FACTOR AS SEPARATE CONSTITUENTS OF A COMPLEX VITAMIN B_2.

By LESLIE JULIUS HARRIS.

From the Nutritional Laboratory, Medical Research Council and University of Cambridge.

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THE observations to be recorded in this paper arose in quite an indirect manner from enquiries in progress in this Laboratory on certain interrelations between vitamin function and redox systems on one side and cell activity on the other. (This work has since been continued in conjunction with Dr S. N. Ray and Dr P. György; and one aspect of it is dealt with in an accompanying paper [Ray *et al.*, 1935].) In the course of these investigations it was observed incidentally that rats fed on certain diets deficient in "vitamin B_2", modelled on the cataract-producing diet of Langston and Day [1933], developed symptoms of severe and characteristic pellagra in a large proportion of cases. As a necessary preliminary to the further prosecution of this work, and also in order to help to clarify the present position with regard to flavin and vitamin B_2, it was thought desirable to examine the nature of the several factors concerned in restoring growth and preventing pellagra on these dietary regimens. As will be shown, the results obtained have confirmed the conclusion of György [1934; 1935] that flavin is not the "rat antipellagra factor". It possesses vitamin B_2 activity in the sense that it is partly responsible for the growth-promotion observed when the usual natural sources of "heat-stable, growth-promoting vitamin B_2" are added to the rat's diet as the supplement to vitamin B_1.

Past work. The growth-promoting action of flavin was demonstrated by Kuhn *et al.* [1933]. They left it undecided whether or not flavin was identical with the antipellagra factor. György [1934] later reported that flavin possesses no antipellagra action for rats. Elvehjem and Koehn [1934] have recently reached the same conclusion using chickens. A number of earlier workers [Sure *et al.*, 1931; Thatcher *et al.*, 1931; Gurin *et al.*, 1931; Sherman and Sandels, 1929; 1931] had in fact suspected that vitamin B_2 (or G) as usually measured might be a complex; but Roscoe [1933] could find no convincing grounds for differentiating between the dermatitis-preventing principle and the factor responsible for growth in various sources of vitamin B_2.

In recent papers Kuhn and his coworkers have used the term vitamin B_2 as though synonymous with flavin; whereas Elvehjem and Koehn [1934] now propose to restrict it to the antipellagra factor. György [1934] on the other hand has proposed to name the antipellagra factor vitamin B_6 and retain vitamin B_2 for flavin.

EXPERIMENTAL.

In the first experiment to be mentioned, begun early in 1934, a group of 20 rats was put on the following diet, similar to that described by Langston and Day.

Extracted caseinogen (Glaxo)	18
Salt mixture	4
Cod-liver oil	2
Butter-fat	8
Maize starch	68

International vitamin B_1 standard, 2 "doses" per rat daily.

After three and a half months no signs of cataract were visible; but six animals had developed severe symptoms of "pellagra".[1] This took the form of a symmetrically disposed intense dermatitis accompanied by a striking scaliness, more especially noticeable on the paws, the nose and the ears. A sticky secretion accumulated in the eyes, the lids remaining half-closed. All animals had ceased to gain weight after the first 4 weeks (except two rats which became spontaneously refected, with characteristic faeces—nos. 7 and 10, Fig. 3—and grew normally and remained free from symptoms without any addition of flavin or antipellagra concentrate, or other source of vitamin B_2 to the diet).

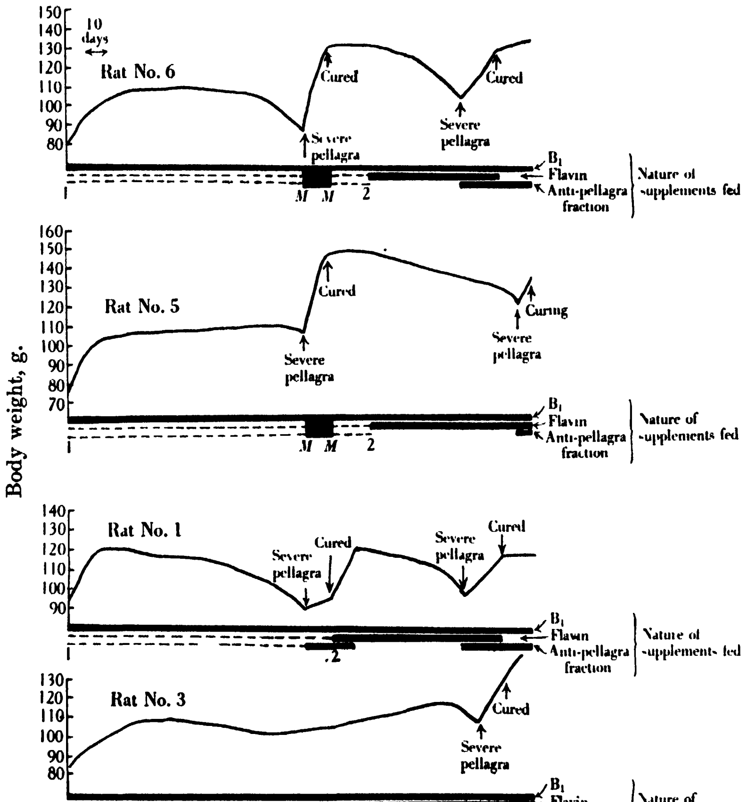

Fig. 1. Effect of different constituents of the vitamin B complex on cure or prevention of "rat pellagra", and corresponding weight curves. From 1 to 2 vitamin B_1 was given in the impure form of the International Standard, contaminated with traces of the other B-factors. After 2 it was given in a highly purified condition. At *MM* the entire complex was given in in the form of marmite.

To test the nature of the deficiencies in this diet and their individual responsibilities for the different symptoms observed, various supplements (concentrates or natural food materials) were fed. Thus, in the first place, two animals were given the complete vitamin B complex in the form of 1·0 ml. daily of a 50 % solution of marmite (laboratory quality). The result was that the pellagrous lesions disappeared in 8–10 days in one animal, and in 12–14 days in the other and growth was resumed (see rats nos. 5 and 6, Fig. 1). The corresponding untreated controls remained uncured.

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[1] Throughout this paper the term "pellagra" is used to describe this condition in rats without prejudice to the question of its identity or otherwise with pellagra in man.

In all later work the activated acid clay (International Standard) was replaced by a purer preparation of vitamin B_1 prepared according to the method of Windaus.[1] This was found to permit the more regular production of symptoms. It may be concluded from this finding that the International Standard contains sufficient traces of the antipellagra factor to delay the regular appearance of symptoms. It may also possibly contain other "B"-factors as impurities.

Apart from purified vitamin B_1, the other active fractions employed were (1) a crystalline preparation of lactoflavin, and (2) the further "supplementary fraction" used by Kuhn *et al.* ("Peters's eluate"). The latter contains the antipellagra principle: it is free from flavin, but contains additional vitamin B_1, and possibly also other "B"-factors.

Results.

To save space, the principal results are summarised in the form of charts (Figs. 1–3) and in Table I.

Table I. *Results of prophylactic and curative tests.*

Nature of supplements given in prophylactic tests	Result	Further additions made in curative tests	Result
Impure vitamin B_1 (as Donath and Jansen's acid clay absorbate) Vitamin B_1	Loss in weight; pellagra in many cases	(1) Flavin	Some improvement in weight but no cure of pellagra
		(2) "Peters's eluate"	Pellagra cured but weight curves still subnormal
		(3) Flavin + "Peters's eluate"	Pellagra cured and weight curves normal
		(4) B complex as marmite	
Vitamin B_1 + flavin	Pellagra in all cases	"Peters's eluate"	
Vitamin B_1 + "Peters's eluate"	No pellagra, but weight curves subnormal	Flavin	
Vitamin B_1 + flavin + "Peters's eluate"	No pellagra; weight curves normal	—	—

DISCUSSION.

The above results confirm the finding of György that flavin does not prevent or cure pellagra in rats. The pellagra-preventing factor is present in the "Peters eluate". For normal nutrition, both flavin and the antipellagra factor must be present simultaneously. Uncomplicated deficiency of flavin (*i.e.* in the presence of vitamin B_1 *plus* the antipellagra supplement) was mainly manifested, in our experiments, by the loss of weight; and the rather unspecific skin lesions reported by György were not a prominent feature.

Vitamin B_2 nomenclature.

According to the definition given by the Committee on Accessory Food Factors [1927] vitamin B_2 is "the more heat-stable, water-soluble dietary factor,

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[1] We are indebted to the I.G. Fabenindustrie of Germany for the supplies of purified vitamin B_1, crystalline flavin and "Peters's eluate".

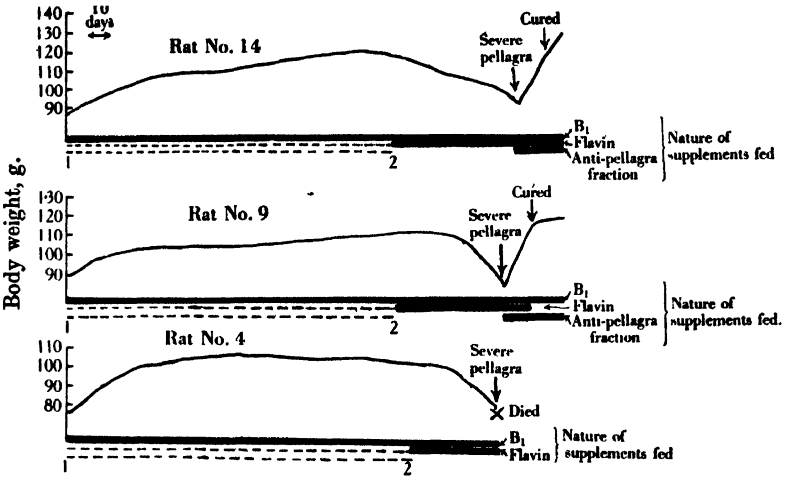

Fig. 2. The dietary supplements are indicated as in Fig. 1.

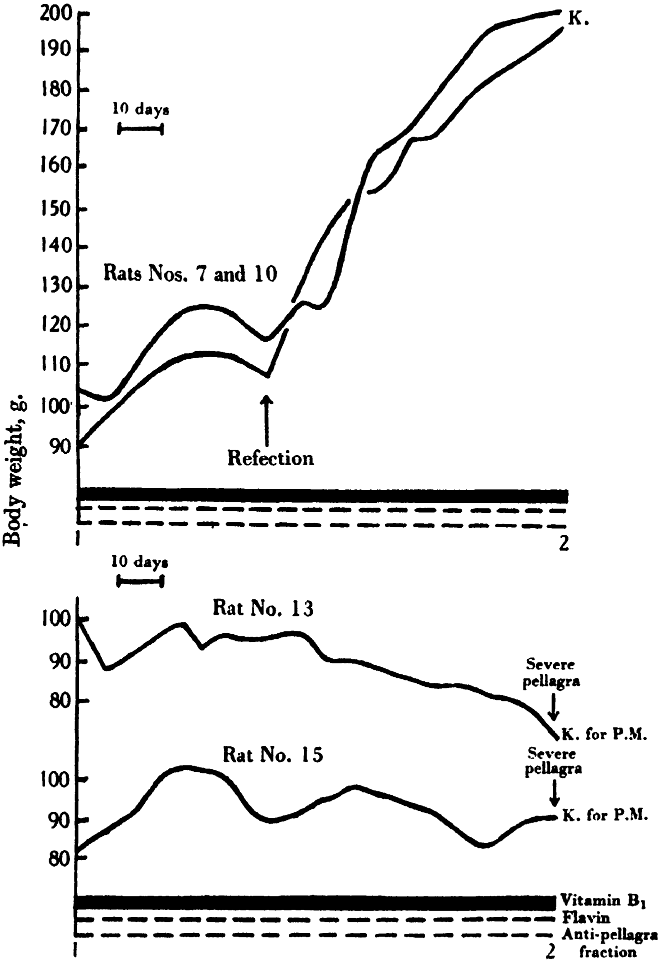

Fig. 3. Above, synthesis of vitamin B_2 complex by refection, with prevention of pellagra. Below, unrefected controls.

recently described and named P-P ('pellagra-preventive') factor by Goldberger, Wheeler, Lillie and Rogers (1926) and found necessary for maintenance of growth and health and prevention of characteristic skin lesions in rats, and considered by the latter workers to be concerned in the prevention of human pellagra". Now, in a large proportion of past experimental work the criterion of vitamin B_2 activity has not been antidermatitis action (since the symptoms of dermatitis were found difficult to produce in experimental animals), but has been rather the restoration of growth. More concretely, vitamin B_2 activity has been measured in actual practice either by the growth-promoting action or by the antidermatitis action of a material such as autoclaved yeast fed as a "heat-stable supplement" to vitamin B_1. In this sense therefore both flavin and the antipellagra factor are constituents of vitamin B_2. Normal growth is impossible in the absence of either flavin or the antipellagra factor.

It has been suggested that the term vitamin B_2 should now be restricted to the antipellagra factor [Elvehjem and Koehn, 1934]. A serious objection to this suggestion is that it would render the bulk of past work (in which growth tests have been the criterion) confusing, if not unintelligible, to future readers. On the other hand it has alternatively been recommended that the term vitamin B_2 should be applied to flavin, and indeed this has already become the current practice in many German papers. This also seems unfortunate, since vitamin B_2 has come to acquire so definite an association with the prevention of pellagra, or pellagra-like lesions. The only logical solution, which will avoid confusion, and render the past literature intelligible, is to retain the term vitamin B_2 for this complex, and to have separate names for the two (or possibly more) constituents. As flavin ("lactoflavin" of Kuhn) already has a chemical name it could be referred to simply as such; or for convenience the two constituents might perhaps be referred to as "vitamins B_2 (F)" and "B_2 (P)", or more shortly vitamins BF and BP; "F" recalling "flavin", and "P" "pellagra" (or rather the "pellagra-like lesions" in experimental animals). (The term "P-P" is already well known and may be kept for the pellagra-preventive factor in man, which further work will probably prove to be identical with the "BP"-factor which prevents the "pellagra-like lesions" in experimental animals.) Thus vitamin BF replaces the vitamin B_2 of Kuhn *et al.*; and vitamin BP replaces the vitamin B_2 of Elvehjem (=the vitamin "B_6" of György; probably identical also with "factor Y" of Chick).

$$\text{vitamin } B_2 \begin{cases} \text{vit. BF } (=\text{flavin} = \text{vit. } B_2 \text{ of Kuhn } et\ al.). \\ \text{vit. BP } (=\text{“pellagra factor”} = \text{vit. } B_2 \text{ of Elvehjem} = \text{vit. } B_6 = (?)\ Y = (?)\ \text{P-P}). \\ \text{—} \end{cases}$$

Final decisions on nomenclature should rest with an international body, such as the Committee on Vitamin Standardisation of the Health Department of the League of Nations. The exact letters chosen do not matter greatly. But in order to preserve continuity in the literature it should be recognised that past work on vitamin B_2 has in fact related to a complex and that new names should therefore be given to the several constituents.

SUMMARY AND CONCLUSIONS.

During the course of experiments on the interrelations between "vitamin B_2" and the redox systems in the eye, rats were fed on a vitamin B_2-deficient diet similar to that used by Langston and Day for the production of cataract, but with the vitamin B_1 provided in the form of the International Standard. It was observed incidentally that about 30 % of these rats developed symptoms of

severe and characteristic pellagra after 3 to 4 months. The opportunity was therefore taken to investigate the antipellagra properties and growth-promoting actions of the different fractions of the vitamin B complex. These were added separately and in different possible combinations, both in curative and in preventive tests, so that their individual functions might be analysed. (In later experiments purer preparations of vitamin B_1 were used and found to lead to the more regular production of symptoms—a result indicating that the International Standard is contaminated with the antipellagra vitamin and possibly with other "B"-factors.) The following conclusions were reached.

(1) *Flavin.* It is confirmed that flavin has no antipellagra action. It is however a constituent of the vitamin B_2 complex in the sense that it causes some improvement in the weight curve when given as a supplement to vitamin B_1. For adequate growth however it was necessary to supplement the vitamin B_1 *plus* flavin with a third fraction, which possesses antipellagra properties. Following Kuhn *et al.* [1933] this was given in the form of "Peters's eluate". With vitamin B_1 and flavin alone all rats without exception developed severe pellagra (eight cases).

(2) *Pellagra-preventing factor.* With vitamin B_1 *plus* the pellagra-preventing fraction only ("Peters's eluate") pellagra was always prevented or cured (thirteen cases). Without the further addition of flavin, however, body weights remained subnormal.

(3) The combined effect of vitamin B_1 *plus* flavin *plus* "Peters's eluate" (containing the antipellagra factor) was approximately equal, in its weight-promoting action and in the cure or prevention of symptoms, to that of the total complex given in the form of marmite.

These results confirm the finding of György that flavin does not possess antipellagra activity. Both flavin and the antipellagra factor should be regarded as separate constituents of the "heat-stable, growth-promoting vitamin B_2 complex".

REFERENCES.

Committee on Accessory Food Factors (1927). Nov. 14th.
Elvehjem and Koehn (1934). *Nature*, **134**, 1007.
Gurin, Eddy, Denton and Ammerman (1931). *J. Exp. Med.* **54**, 421.
György (1934). *Nature*, **133**, 498.
—— (1935). *Biochem. J.* **29**, 741.
Kuhn, György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 317.
Langston and Day (1933). *Southern Med. J.* **26**, 128.
Ray, György and Harris (1935). *Biochem. J.* **29**, 735.
Roscoe (1933). *Biochem. J.* **27**, 1537.
Sherman and Sandels (1929). *Proc. Soc. Exp. Biol. Med.* **18**, 193.
—— —— (1931). *J. Nutr.* **3**, 395.
Sure, Kik and Smith (1931). *Proc. Soc. Exp. Biol. Med.* **28**, 498.
Thatcher, Sure and Walker (1931). *Arch. Path.* **11**, 425.

XC. THE NITRATE CONTENT OF ANIMAL TISSUES, AND THE FATE OF INGESTED NITRATE.

By MARY WHELAN.

From the Department of Biochemistry and Pharmacology School of Medicine, University of Oklahoma.

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Previous work with ammonium nitrate has shown that it is not completely excreted. In man approximately 80 % is excreted by the kidney during the period of intake. The total excretion may reach 90 % since nitrate continues to be excreted for several days after ingestion is discontinued. In dogs the percentage is not nearly so high. On an average, about 70 % is excreted by the kidney. The work of Keith *et al.* [1930] has shown that the amount of nitrate excreted by man in the faeces is negligible. That this is true in the case of dogs has not been proved. In future work on this problem we shall check that point. The fate of nitrate retained in the body is unknown. Nitrate taken by mouth appears quite soon in the blood stream, and analyses of oedema [Keith *et al.* 1930] and ascitic fluids show that it appears in these fluids in concentrations closely approaching that of the serum. Because of the ease with which it diffuses it comes in contact with all tissues. In an effort to determine what becomes of the nitrate retained this study was undertaken.

Method of study. Four dogs six weeks old and of the same litter were used. They were placed on a diet of 250 g. of prepared dog food per day. This was supplemented by a pint of milk and 500 ml. of water. Analysis showed that the nitrate content of food, milk and water was not appreciable. That this was an adequate diet is shown by the fact that the dogs gained weight steadily, on an average 1·7 kg. per dog during the 23 days of the experiment. The dogs receiving no nitrate gained 2 kg. each; the two receiving ammonium nitrate gained 1·4 kg. each. The exact significance of the difference is not apparent but it may be due to dehydration resulting from ingestion of such a large quantity of ammonium nitrate.

The dogs were kept on this diet for 7 days as a control period, and then dogs 1 and 2 were given 10 ml. of 10 % ammonium nitrate per day, by stomach tube, for 16 days. On the twenty-fourth day, 24 hours after the last dose of nitrate, the dogs were killed. This was done by inserting a cannula into the femoral artery, under local anaesthesia, and producing death by bleeding. This removed practically all the blood, as shown by the fact that little bleeding occurred when the tissues were removed. As soon as death had occurred the tissues and organs desired for analysis were removed and weighed. Each organ was then cut into small pieces and mixed, and representative samples were selected for determination of water and chloride contents. The remainder of the tissue was treated with sufficient sodium hydroxide to ensure an alkaline reaction throughout the drying process, and was then placed in a drying oven at 100° until thoroughly dry. It was then ground in a food chopper with a fine knife and was ready for analysis. The nitrate content was determined according to the technique outlined elsewhere [1935]. Chloride content was determined by the method of Wilson and Ball [1928]; the water content, by drying to constant weight. All determinations were made in duplicate and some of the nitrate determinations were made in triplicate. Nitrate in blood and urine was determined according to the method of Whelan [1930].

During the entire period of the experiment, the dogs were kept in metabolism cages and the urine collected under toluene. On each twenty-four hour specimen, volume, p_H and nitrate-nitrogen were determined (Table II). Blood was analysed at intervals for chloride and nitrate-nitrogen (Table I).

Table I. *Chloride and nitrate-nitrogen content of blood.*

mg./100 ml.

	Dog 1		Dog 2		Dog 3		Dog 4	
Date	Cl	NO_3-N	Cl	NO_3-N	Cl	NO_3-N	Cl	NO_3-N
19. iii	361	0	396	0	410	0	370	0
26. iii	386	5·3	390	5·5	—	—	—	—
4. iv	408	6·2	408	5·2	420	0	400	0

Table II. *Chloride and nitrate-nitrogen of urine.*

	Urine		Chlorine		Nitrate-N		
Date	Average volume	p_H range	g./100 ml. average	Total g. average	g./100 ml. average	Total g. average	Dog
12–19. iii	184·4	5·8–6·5	0·408	0·7504	0	0	1
19. iii–4. iv	319·7	6·5–5·2	0·350	1·211	0·0305	0·091	1
12–19. iii	95	5·8–6·5	0·485	0·459	0	0	2
19. iii–4. iv	239·4	6·5–5·2	0·348	0·835	0·0322	0·077	2
12–19. iii	139	5·7–6·5	0·509	0·650	0	0	3
19. iii–4. iv	340	6·3–6·9	0·379	1·29	0	0	3
12–19. iii	348	6·0–6·9	0·358	1·245	0	0	4
19. iii–4. iv	264	6·3–6·2	0·363	0·96	0	0	4

Discussion. As has been repeatedly observed, the ingestion of ammonium nitrate increased the average daily excretion of urine and decreased the p_H. The urine remained negative for nitrate during the control periods, and in the case of the dogs receiving no nitrate remained nitrate-free during the entire period of the experiment. The average daily excretion of nitrate-nitrogen during the period of ingestion in dogs 1 and 2 was 0·0305 and 0·0322 g. respectively. Although the concentrations were similar, less total nitrate was excreted by dog 2 than by dog 1. Dog 1 excreted 53·8 % of that ingested and dog 2, 45·4 %. At the time of death approximately half of the nitrate had not been excreted. The possibilities are: (1) that it was all retained and had been taken up by the various tissues, possibly replacing chloride; (2) that it had been destroyed, by being broken down into some form which does not produce a colour reaction with diphenylbenzidine; (3) that it entered into organic combination of some type, which is not altered by treatment with acid, and therefore cannot be extracted with ether; (4) that it is not completely absorbed from the gastro-intestinal tract. Possibilities (2) and (3), as will be shown later, are substantiated by data.

Analyses of tissues.

Chloride. Chloride determinations (Table III) were made on all tissues. The results were variable and seemed to bear no relation to nitrate ingestion. The table is included because the variability of results may have some bearing on the analysis of the problem.

Nitrate. Table IV gives the results of analyses of tissues for nitrate-nitrogen. In all cases, with the exception of the oesophagus, the nitrate contents of the tissues of dogs 1 and 2 were markedly higher than those of the corresponding tissues of dogs 3 and 4. Taking the average difference between the tissues of dogs which had received nitrate and of those which had received none shows that the greatest difference occurs in trachea, pancreas, liver, spleen, kidney and muscle. Of these, the largest differences occurred in trachea, pancreas and liver; the least differences occurred in the oesophagus; the urinary bladder and intestine also showed very small differences. In the other tissues, namely brain, spinal cord, lung, heart, stomach and skin, the difference in nitrate content between control and nitrate-fed dogs is marked and almost identical in all cases.

Table III. *Chloride content of tissues.*

Chloride g./100 g.

Tissue	Dog 1	Dog 2	Dog 3	Dog 4
Pancreas	0·137	0·172	0·153	0·168
Heart	0·128	0·143	0·128	0·103
Kidneys	0·257	0·246	0·256	0·242
Spleen	0·172	0·149	0·168	0·157
Trachea	0·179	0·178	0·226	0·185
Oesophagus	0·135	—	0·126	—
Bladder	0·191	0·149	0·216	0·197
Brain	0·162	0·171	0·164	0·153
Spinal cord	0·153	0·179	0·199	0·186
Lung	0·231	0·217	0·186	0·192
Liver	0·131	0·106	0·122	0·102
Stomach	0·123	0·193	0·173	0·108
Intestine	0·090	0·126	0·101	0·084
Muscle	0·113	0·108	0·120	0·128
Skin	0·211	0·212	—	0·184

Table IV. *Nitrate-nitrogen content of tissues.*

Nitrate-N mg. per 100 g. dry tissue.

	Received nitrate						
Tissue	Dog 1	Dog 2	Dog 3 Control	Dog 4 Control	Average 1–2	Average 3–4	Average difference
Brain	1·03	1·00	0·60	0·57	1·02	0·59	0·43
Spinal cord	1·39	1·30	0·96	0·86	1·35	0·91	0·44
Trachea	1·86	1·51	0·50	0·43	1·69	0·47	1·22
Lung	0·97	1·20	0·53	0·73	1·09	0·63	0·40
Heart	1·35	1·39	0·82	0·81	1·38	0·82	0·46
Stomach	1·15	0·85	0·37	0·40	1·00	0·53	0·47
Pancreas	1·58	1·64	0·61	0·53	1·61	0·57	1·04
Liver	1·43	1·50	0·65	0·64	1·47	0·65	0·82
Spleen	1·96	1·50	0·40	0·60	1·73	0·50	1·23
Kidney	1·15	1·46	0·67	0·77	1·32	0·72	0·60
Intestine	1·00	0·70	0·63	0·32	0·85	0·48	0·37
Muscle	1·86	1·40	0·84	0·74	1·63	0·84	0·79
Oesophagus	1·24	—	1·21	—	1·24	1·21	0·03
Skin	1·46	1·11	0·78	0·95	1·29	0·87	0·42
Bladder (urinary)	1·17	1·28	1·00	—	1·23	1·00	0·23

Table V gives the weights of fresh tissues, the percentage of water in each, and the calculated concentration of nitrate-nitrogen in the wet tissues as well as the total nitrate-nitrogen content of each. It emphasises the facts brought out

Table V. *Nitrate-nitrogen content of wet tissue.*

	Dog 1	Dog 1 Nitrate-N			Dog 2	Dog 2 Nitrate-N			Dog 3	Dog 3 Nitrate-N			Dog 4	Dog 4 Nitrate-N		
Tissue	wt. g.	mg./ 100 g.	mg. total	H_2O %	wt. g.	mg./ 100 g.	mg. total	H_2O %	wt. g.	mg./ 100 g.	mg. total	H_2O %	wt. g.	mg./ 100 g.	mg. total	H_2O %
Brain	63·2	0·176	0·112	82·8	60·88	0·186	0·113	81·4	72·89	0·106	0·077	82·3	75·87	0·105	0·080	81·6
Spinal cord	10·7	0·317	0·034	77·2	10·38	0·385	0·037	72·4	11·37	0·263	0·030	72·6	9·68	0·240	0·022	72·1
Trachea	15·21	0·586	0·090	68·4	11·46	0·384	0·044	74·6	15·67	0·113	0·017	77·7	14·29	0·103	0·015	76·0
Lungs	47·53	0·201	0·096	79·3	57·79	0·242	0·140	79·8	53·39	0·110	0·058	79·2	54·79	0·158	0·087	78·3
Heart	34·58	0·230	0·090	79·4	35·62	0·295	0·105	78·8	32·08	0·161	0·052	80·3	33·85	0·169	0·057	79·4
Stomach	60·90	0·214	0·131	81·35	50·12	0·173	0·087	79·6	60·73	0·083	0·051	77·6	75·13	0·063	0·047	84·3
Pancreas	14·93	0·296	0·044	81·3	11·5	0·323	0·037	80·3	13·53	0·147	0·020	75·9	13·97	0·142	0·020	73·3
Liver	218·43	0·343	0·750	76·0	197·18	0·402	0·770	73·2	224·62	0·184	0·413	71·7	241·00	0·154	0·372	71·0
Spleen	8·94	0·406	0·036	79·3	11·34	0·316	0·036	79·0	8·015	0·074	0·006	81·5	10·49	0·127	0·013	78·8
Kidneys	38·92	0·230	0·090	77·0	34·37	0·314	0·108	78·5	42·48	0·138	0·059	79·4	41·66	0·172	0·072	77·7
Intestine	301·2	0·166	0·500	83·45	253·88	0·155	0·392	77·8	312·2	0·117	0·365	81·35	356·9	0·060	0·215	81·2
Muscle	2482·0	0·512	12·708	72·5	2230·0	0·356	7·950	74·6	2570·0	0·211	4·94	74·8	2660·0	0·206	5·500	72·3
Oesophagus	15·62	0·279	0·044	77·5	—	—	—	—	20·31	0·235	0·048	80·6	—	—	—	—
Skin	950·0	0·806	7·660	44·7	840·0	0·534	4·480	52·0	968·0	—	—	—	998·2	0·60	5·980	37·0
Bladder	3·5	0·377	0·0132	67·8	3·25	0·400	0·013	68·7	5·34	0·231	0·012	76·9	4·18	—	—	72·7
Blood	406·0	6·2	25·200	—	364·0	5·2	18·90	—	420·0	—	—	—	434·0	—	—	—
Dog	5800·0	—	—	—	5200·0	—	—	—	6000·0	—	—	—	6200·0	—	—	—

in Table IV, but the values shift somewhat because of differences in water content. In this table are included the estimated total contents of muscle, skin and blood. The calculations were made by using the figures given by Skelton [1927] for the proportion of the weight of the organs to the total body weight.

Table VI gives the total values for nitrate-nitrogen ingested, excreted and retained, as well as total amounts determined in the tissues. The nitrate content of tissues of the dogs which received nitrate was six to eight times higher than that of those which did not receive nitrate. Dog 2 excreted a smaller percentage of nitrate than dog 1, and the nitrate content of the total tissues was also smaller. But the differences are slight in comparison to the total amount of nitrate retained.

Table VI. *Relationship between nitrate-nitrogen ingested, excreted and retained.*

	Dog 1 mg.	Dog 2 mg.	Dog 3 mg.	Dog 4 mg.
Nitrate-N ingested	2700	2700	0	0
Nitrate-N excreted	1452	1225	0	0
Nitrate-N retained	1248	1475	0	0
Nitrate-N in tissues	47·59	33·21	6·15	10·18
Nitrate-N in tissues per kg. body weight	8·21	6·39	1·02	1·66

This shows, then, that nitrate retained in the body is either destroyed or enters into some organic combination which is not broken down by the treatment to which we have subjected these tissues. Upon this theory we are undertaking some further work on this problem. There is also the possibility that nitrate taken by mouth by dogs is not completely absorbed from the gastro-intestinal tract. From experimental work on human subjects, it would seem that this is quite unlikely. However, the possibility of this fact accounting for the loss of 50 % of the total amount seems to be very remote. As shown in Table VI, the amount of nitrate which can be accounted for in the tissues is less than 4 % of the difference between the amount ingested and that excreted in the urine. This leads us to the conclusion that nitrate is destroyed or altered by the tissues and therefore accounts for the fact that it is not recovered quantitatively in the urine.

Table VII shows that only 75 % of the nitrate-nitrogen added to the dry tissue *in vitro* can be recovered. But pure potassium nitrate added to the same amount of asbestos fibre as is used in all extractions can be completely recovered.

Table VII. *The recovery of nitrate added to tissues.*

Weight of sample g.	NO_3-N in sample mg.	NO_3-N added mg.	NO_3-N determined mg.	NO_3-N recovered mg.	% recovered
2	0·0275	0·05	0·055	0·0375	75
2	0·0169	0·05	0·057	0·0386	77·5

SUMMARY.

1. The nitrate-nitrogen content of animal (dog) tissues is given. Tissues analysed were taken from dogs which had received no additional nitrate, as well as from dogs which had received large amounts of ammonium nitrate. The latter showed an increase of six to ten times the content of normal tissues.

2. Approximately 50 % of the nitrate-nitrogen ingested was excreted by the kidney.

3. Although it has not been shown experimentally in dogs, it seems unlikely that lack of absorption from the gastro-intestinal tract explains the recovery of only 50 % in the urine.

4. Analyses showed that only a small part is taken up by the tissues and retained as inorganic nitrate.

5. The findings in this study suggest that nitrate is destroyed in the tissues and this explains why it cannot be recovered quantitatively in the urine.

REFERENCES.

Keith, Whelan and Bannick (1930). *Arch. Int. Med.* **46**, 797.
Skelton (1927). *Arch. Int. Med.* **40**, 140.
Whelan (1935). *J. Lab. Clin. Med.* (in press).
—— (1930). *J. Biol. Chem.* **86**, 186.
Wilson and Ball (1928). *J. Biol. Chem.* **79**, 221.

XCI. A PRELIMINARY NOTE ON THE APPEARANCE OF PARESIS IN ADULT RATS SUFFERING FROM CHRONIC AVITAMINOSIS E.

By AXEL RINGSTED.

From the University Institute of Hygiene and the State Vitamin Laboratory, Copenhagen, Denmark.

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This paper deals with the observation of some characteristic neuropathic disturbances which have developed in adult albino rats fed on various vitamin E-free food mixtures. Similar observations have not, to my knowledge, been previously dealt with, though Evans and Burr [1928] have observed the appearance of paresis in suckling rats bred from vitamin E-free mothers.

Evans and Burr [1928] found that the progeny of mothers fed on a diet very poor in vitamin E developed paresis in the hind quarters during the latter part of the lactation period. The paresis appeared as a rule on about the 20th day after birth and always before the 25th day. Otherwise the animals did not become paretic. An initial relaxed paretic stage appeared in some of the animals, whilst the majority showed signs of spasticity in the hind legs from the outset. A claw-like condition of the feet developed later. No disturbance of sensibility was evident. In the course of the following months, symmetrical baldness of the hind quarters and femur and atrophy of the skin occurred. Paresis spontaneously disappeared in scarcely 20 % of the diseased animals. Some individual animals suffering from a mild attack of paresis were cured by a liberal supply of substances containing vitamin E. The great majority appeared to be quite unaffected by treatment with various vitamins—even vitamin E.

Evans and Burr were able to show, by means of a wide range of experiments, that this paresis was due to lack of vitamin E in the diet, and that other organic and mineral ingredients of the food had, *per se*, no direct causal significance in the appearance of paresis. The experiments showed that it was possible completely to prevent the appearance of paresis by giving the mothers, prophylactically, substances rich in vitamin E, such as yellow corn, corn oil and wheat germ oil. It was evident that it was the vitamin E component of wheat germ that was the conclusive factor in experiments with many different fractions of wheat germ oil. These experiments showed a quantitatively exact agreement in the properties of the wheat germ in curing sterility and preventing paresis.

Evans and Burr's observation on this paresis was later confirmed by Mason [1933]. Mason succeeded in producing this paralysis in suckling rats but not in keeping the latter alive. He is of the opinion that that was due to the difficulty of finding just the right quantity of vitamin E which is sufficient to maintain life and insufficient for the vigorous development of the nervous system in the lactation period.

Evans and Burr and Mason give no information as to the morbid anatomical changes in the nerve system of these paretic rats.

Experimental methods.

The animals used for experiment comprised three groups of albino rats, A, B and C, 27 animals in all, 26 female and 1 male. The mothers of these animals had all had a uniform diet, Gudjonsson's diet 4 for breeding animals. The animals were used for the experiment at the age of 24–35 days (see Tables II, III, IV). The cage of each rat was provided with a wire mesh base so that the faeces fell through.

The rats were constantly supplied with fresh drinking water, were given fresh food three times a week and were weighed once a week, and during pregnancy every day.

From puberty, a vaginal smear was taken six times a week from all the females. The purpose of this was to ascertain the regularity of the period of oestrus; to decide the optimum time for copulation; to find out whether copulation had taken place (bouchon vaginale, sperm in the vaginal smear) and whether conception had occurred (placental sign). The vaginal smear provides, furthermore, information as to the period of the cessation of the resorption-pregnancy and as to the reserves of vitamin A in the organism (see below).

The rats were kept at a constant temperature of 22°. No sign of acute or chronic infection was ever observed among the animals that were killed. The composition of the food mixtures, the content and method of administering the vitamin preparations and also their preservation and manner of distribution are given in the two following sections.

As to the composition of the breeding diet and further data as to fertility and the suitability of the experimental animals for vitamin A experiments, the reader is referred to Gudjonsson [1930, 1, 2].

Table I. *Food mixtures.*

Group of animals ...	A	B	C
	Vitamin E-free I	Vitamin E-free III	Vitamin E-free II
Food mixture	%	%	%
Caseinogen	18	20	20
Rice starch	50	53	53
Oxidised lard	20	15	15
Dried autolysed top yeast	7	7	7
Salt mixture (185)	5	5	5
Daily additions of vitamin A	Cod-liver oil	Carotene	Cod-liver oil
Daily additions of vitamin D	Cod-liver oil	Vit. D-standard	Cod-liver oil

The vitamin E-free food mixtures may all be taken together under the same heading.

Caseinogen. To avoid any possibility of its containing vitamin E, ether-extracted caseinogen was used during the first 1½ months of the experiment. As contemporaneous experiments showed that caseinogen treated with acid did not contain any appreciable quantity of vitamin E, this was used for the rest of the experiment.

Rice starch was an ordinary commercial product, as also was the lard. The *lard* was oxidised by being kept at a temperature of 110° for 10 hours with simultaneous aeration. This prevented the possible presence of vitamins A and E.

McCollum's salt mixture (185) was used.

At first, a peanut oil solution of a biologically standardised cod-liver oil was used as a source of *vitamin A* for Group A, so as to give a daily dose of 8 I.U. of vitamin A. As some of the rats about 8–12 weeks old evinced a prolonged period of oestrus[1], up to 3–4 days' duration, the daily dose was increased to 40 I.U. of vitamin A of fresh standardised cod-liver oil, after which this phenomenon disappeared.

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[1] Evans and Bishop [1922] showed that at the commencement of deprivation of vitamin A the period of oestrus—confirmed by the vaginal smear—became longer and dioestrus shorter. Later on the stage with cornification becomes continuous. This was subsequently confirmed by Hohlweg und Dohrn [1930] and Aberle [1934].

From the beginning of the experiment the animals in Group B were given 20γ of standard carotene dissolved in sesame oil daily.

The same cod-liver oil solution in the same dose as mentioned for Group A was used for Group C from the commencement of the experiment, *i.e.* 40 I.U. of vitamin A.

Dried autolysed top yeast mixed with the diet so as to form 7 % of it was used as a source of *vitamin B* for all three groups.

The yeast was autolysed at 38° for 24 hours, after which it was dried on flat trays at 30° and then powdered. As the increase in weight began to diminish, certain of the animals were given an addition of 0·5 g. dry yeast for a longer period.

We gave no *vitamin C*, as rats do not need this supplied in their food.

The animals in Group A received in their daily addition of cod-liver oil 0·4, and later 2, I.U. of vitamin D, those in Group C had 2 I.U. of vitamin D throughout the whole experiment. A sesame oil solution of standard vitamin D was used for Group B, mixed with food so that the mixture contained 0·8 I.U. of vitamin D per 10 g. of food.

The vitamin E content of the food mixture is certainly very low or nil. The phenomenon of initial fertility, which is due partly to the presence of reserves of vitamin E and partly to the food containing small quantities of vitamin E, never appeared in animals fed on these food mixtures. Even animals that became pregnant after having only been on the experimental diet for 20–30 days had so exhausted their vitamin E reserves that they resorbed the foetus. The reason for the low content of vitamin E in the diet lies certainly in the thorough oxidation of the lard by which the whole of the vitamins A and E are effectively destroyed [Fridericia, 1924; Mattill and Crawford, 1931; Cummings and Mattill, 1930].

We lay the greatest stress on the very low vitamin E content of the diet which is certainly of decisive significance for the appearance of paresis (see later).

A preparation of wheat germ oil "*Fertilan*"[1] was used as a source of vitamin E. The wheat germ oil was kept in hermetically sealed ampoules at 0°. The contents of the ampoule were warmed and shaken up before use and kept at 0° after use again. An ampoule lasted for 2–3 days. The oil was measured with the same standard pipette for all the experimental groups. One drop weighed on an average 25·4 mg.

Clinical observations.

The animals in the three groups, except No. 58, throve well for the first 8–12 weeks.

At about the 10th week of the experiment the rate of increase in weight appreciably diminished and in spite of a doubling, shortly followed by a quadrupling, of the daily dose of cod-liver oil, the animals put on weight very slowly. The average weight curve of the animals was, meanwhile, very near the average weight curve for rats of the same breeding fed on "diet 4 for breeding animals" [Gudjonsson, 1930, 2] (Fig. 1).

The females were mated with normal males between the 80th and the 100th experimental days. All pregnancies resulted in resorption. The females were, thereupon, available for use as test animals and were used for investigation as to the vitamin E content of various substances, by the curative method exclusively [Evans and Burr, 1927]. Ten females (see Tables II, III, IV) were given varying quantities of wheat germ oil at different periods after conception had been verified. They all gave birth to live progeny except No. 55 which was first given wheat germ oil on the 7th day of pregnancy[2] and No. 78 which had

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[1] "Fertilan" wheat germ oil was very kindly supplied to us by the Danish Medicinal Company's Experimental Laboratory. For information as to its preparation reference may be made to Vogt-Møller's [1931] articles on this subject.

[2] This confirms Evans and Burr's observation that vitamin E should be given before the 5th or 6th day after conception has taken place so that the fertile ovum may develop fully.

had too small doses. None of these 10 animals later showed signs of neuropathic disturbances.

The 16 other females were used for investigation of the vitamin E content of various foodstuffs. All these and the only male later developed paresis.

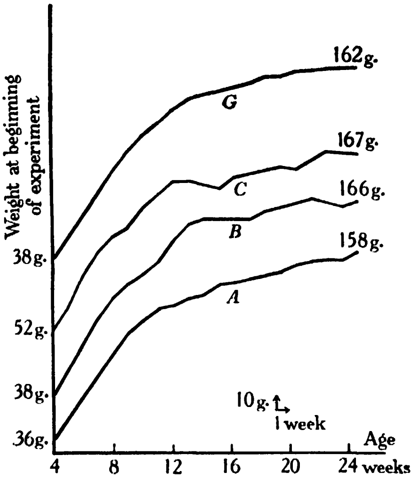

Fig. 1. Average weight curves for the first 20 experimental weeks for animals in groups *A*, *B* and *C* compared with Gudjonsson's average weight curve (*G*) for female rats on "diet 4 for breeding animals".

As the details concerning these tests have no relation to the pareses described later on they are left out of this preliminary communication.

It should however be pointed out that the vitamin E content of the substances was so low that all the pregnancies resulted in resorption. In about the 19th week of the experiment it was noticed that one animal (No. 58) was beginning to drag its hind legs a little. Simultaneously the legs began to straddle slightly. About 8 weeks later, when similar disturbances in gait were observed in three other rats (Nos. 51, 53 and 56), the conclusion was come to that this was no accidental phenomenon.

It was further confirmed by a closer examination of the gait of all the animals in Groups B and C during the following weeks. It appeared that several of these animals had already begun to show disturbances in their gait. We were hereby in a position to observe the very first stage in the development of the disease.

These observations have provided the basis of the following description in which, for practical reasons, the disease has been divided into three stages, or grades, as all the animals did not develop the disturbances to the highest degree, even though they continued on the diet for several weeks after the appearance of the first symptoms.

Stage I. The first clinical symptoms are a dragging of the legs and slight degree of inco-ordination in the gait. The gait is slower and a little cautious and the feet are placed slightly out to the side[1]. The strength of the hind legs is good, and the animal can jump with ease (7–8 cm.). In some cases the fur has begun to loosen on the hind quarters and the hind legs. The animal is lively and eats well. This condition changes with no definite break into stage II.

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[1] The disturbance in the gait is most evident when the animal is allowed to walk over the wide-meshed wire netting of the roof of the cage.

Table II. *Group A. Vitamin E-free diet I.* (*Female rats.*)

Serial no.	Litter no.	Age at commencement of exp. days	Addition of W.G.O. during 1st test pregnancy	Time of appearance of paresis weeks	Stage of paresis	Treatment of paresis	Result of treatment
50	1135	26	+	—	—	—	—
51	1135	26	-	30	II	1000 I.U. of vit. A daily for 4 weeks 50 I.U. of vit. D daily for 4 weeks	Continued development of paresis to stage III
52	1136	26	+	—	—	—	—
53	1136	26	-	27	III	125 mg. W.G.O. daily for 12 weeks	No effect
54	1136	26	+	—	—	—	—
55	1136	26	+	—	—	—	—
56	1136	26	-	27	II	None*	—
58	1134	29	-	19	III	1 g. autolysed dried top yeast daily for 6 weeks	No effect
59	1134	29	+	—	—	—	—
61	BfR2	23	+	—	—	—	—

Table III. *Group B. Vitamin E-free diet III.* (*Female rats and one male.*)

75	1810	26	-	24	II	None	—
76	1810	26	+	—	—	—	—
77	1810	26	-	24	I	600 I.U. of vit. A daily for 3 weeks 30 I.U. of vit. D daily for 3 weeks	Continued development of paresis to stage II
78	1810	26	+	—	—	—	—
79	1810	26	-	21	II	*ca.* 100 mg. W.G.O. daily for 4 weeks	No effect
80	1810	26	-	20	III	None	Condition worse
82	1146	24	+	—	—	—	—
83	1146	24	-	20	I	1·5 g. autolysed yeast daily for 3 weeks	Continued development of paresis to stage II
84	1830	25	-	23	I	1·0 g. autolysed dried yeast daily for 6 weeks	Continued development of paresis to stage III
85	1830	25	-	20	III	80 I.U. of vit. A daily for 30 days 4 I.U. of vit. D daily for 30 days	No effect

Table IV. *Group C. Vitamin E-free diet II.* (*Female rats.*)

88	1147	35	+	—	—	—	—
89	1147	35	-	22	I	1 g. autolysed dried yeast for 6 weeks	Increased baldness and paresis to stage II
90	1147	35	-	23	I	75 mg. W.G.O. daily for 6 weeks	No effect
91	1147	35	-	34	I	None	—
103	BfR46	24	-	16	II	None	—
104	BfR46	24	-	17	1	50 mg. W.G.O. daily for 6 weeks	No effect
105	BfR46	24	-	15	II	250 mg. W.G.O. daily for 6 weeks	No effect

* Nos. 57 and 60 were killed during the first test pregnancy long before the paresis had appeared. No. 56 was operated on shortly after the commencement of paresis and died a few days after the operation with symptoms of ileus.

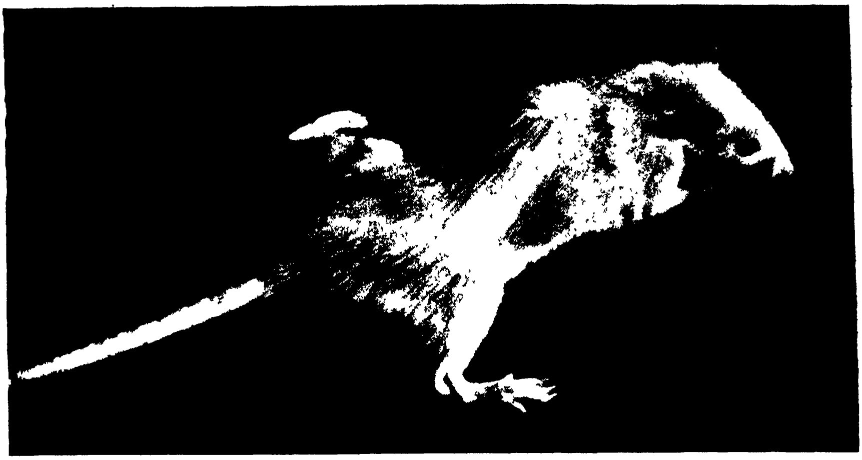

Fig. 2. BfR. 58. Noticeable straddling of the hind legs. Low position of the body. Stage II.

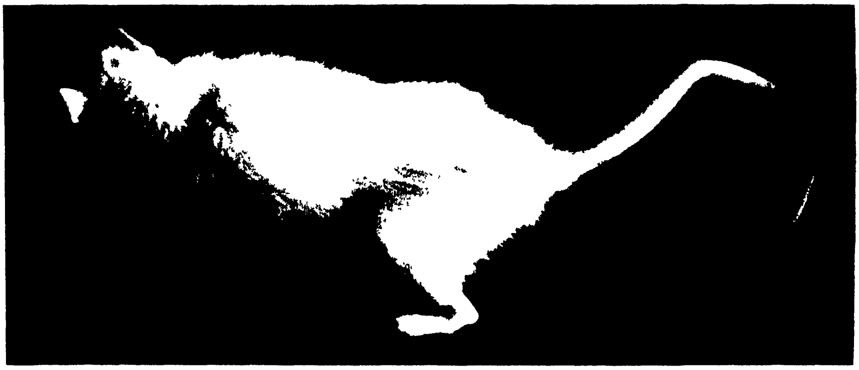

Fig. 3. BfR. 58. Noticeable straddling. Soles of the feet flexed. Toes flexed. The animal treading on the lateral edge of the foot.

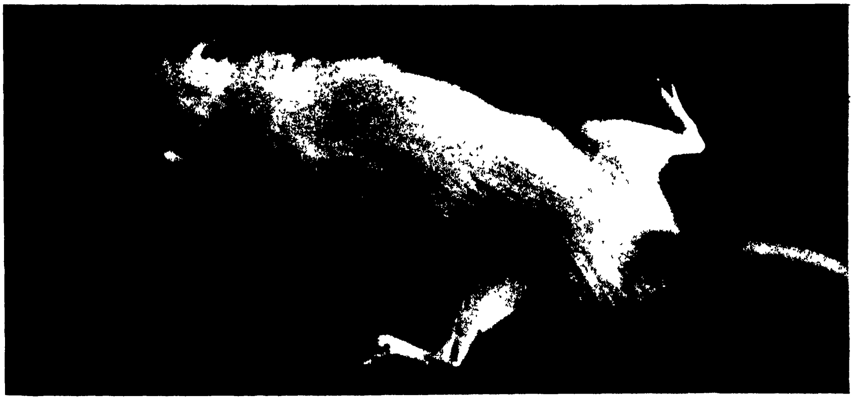

Fig. 4. BfR. 85. Hind quarters atrophied. Baldness most pronounced on the legs. Distinct straddling gait. Hind quarters dragging along the floor. Flexion of feet and toes. Stage III.

Stage II. This stage (Figs. 2 and 3, Plate I) is distinguished by a pronounced straddling of the hind legs when walking. A healthy rat keeps the foot under the femur so that it is covered by the latter when seen from above [Aberle,1934]. In this stage there is a considerable diminution in the functions of the adductors. There is flexion of the sole of the foot and extension of the knee joint. The inco-ordination is pronounced. The animals frequently walk upon the side of the foot (Fig. 3) and on the tail without noticing it. Paresis of the adductor is clearly apparent when the animals are taken out of the small opening in the cage. They must be taken out in a slanting position as, otherwise, the straddling leg is caught. When moving, the hind quarters are kept low. There is incipient atrophy of the muscles of the hind quarters and hind legs. The fur begins to fall out, especially from the extremities, but to a less degree in the centre. Susceptibility to touch seems also to be lessened. If the tail and hind legs be taken in a pair of forceps, there is an impression that there is hypoaesthesia in most of the animals in this stage and hypoalgesia in many of them.

The fore legs seem to be quite normal at this stage. The animals are lively, but move round the cage slowly.

Stage III. The gait is more characteristic in this stage (Fig. 4, Plate I). The hind legs are kept very highly abducted, and cannot be adducted. The hind quarters are dragged along the floor (Fig. 4). The animals in reality move forwards on their stomachs. The hind quarters and tail swing from side to side at every step. The sole of the foot is highly inflexed when walking, as are the small joints of the toes. If the animals are taken up by the nape of the neck, there is no dorsal flexion of the foot, as is the case with normal rats [Aberle, 1934]. Ataxia is still more pronounced and gives rise to the most grotesque attitudes and attempts to walk. These phenomena were most pronounced when the rat tries to jump up in the cage and in walking tests on the wide-meshed netting roof of the cage. There is anaesthesia or hypoaesthesia of the tail and hind legs and nearly always hypoalgesia of the tail. The fur is thinned out to a very great extent, mostly on the legs and nates, to a less degree on the other parts of the hind quarters. In most cases the baldness was found to be diffused symmetrically. The animals are less lively and constantly sit quietly in a corner and sleep. Their appetite is diminished and a slow falling off in weight of some few g. a week is noticed in a few of them.

Therapeutic experiment.

As part of the attempt to find the causes of the neuropathic disturbances, therapeutic experiments were carried out with large doses of vitamins A, B, D and E. Animals in different stages were selected, as well as those which had shown the symptoms for various lengths of time. The details of dosage and the results obtained are shown in Tables II, III and IV.

Since all the observations indicated that animals that had been given a potent preparation of vitamin E were protected against paresis, it seemed also desirable to investigate whether the paretic syndrome was affected by wheat germ oil. Treatment was begun in four animals (Nos. 53, 90, 104, 105) 2–4 weeks after the paresis was observed. Unfortunately there was no more of the same wheat germ oil as was used earlier on in the experiment. The new preparation, which was also called "Fertilan" was prepared in a different way—not otherwise further elucidated [Vogt-Møller, 1934]. It was supposed to be of the same strength as the wheat germ oil which had been given previously in the experiment. From later experiments, however, it has shown itself to be ineffective with regard to the preservation of pregnancy in doses of as much as 20 drops (500 mg.) daily.

As a result of these experiments we may conclude that large doses of vitamins A, B and D have no curative effect on the paresis, while nothing definite can be said as to the effect of vitamin E therapy.

DISCUSSION.

The clinical phenomena which have been described have a general similarity to the neuropathic disturbances in rats suffering from vitamin A deficiency described by Aberle [1934] and to the phenomenon in suckling rats from mothers on a diet low in vitamin E described by Evans and Burr [1928] but differ from them in essential points. The characteristic in the paralysis in the suckling young [Evans and Burr, 1928] is the initial stage with a sudden development of a spastic paralysis of the hind legs, feet and toes in a period of pronounced growth, from the 20th to the 25th days. The few that primarily showed a relaxed paresis also ended in a spastic condition.

All my paretic animals were almost adult when the paresis set in. The paresis was of the relaxed type. It developed slowly so that it was possible to differentiate the various stages. The paresis was accompanied by disturbances of the sensibility affecting especially the deep-seated and to a less degree the cutaneous sensibility of the legs and tail.

Since the vitamin E preparation given in our therapeutic experiment was shown to be quite inactive—in some control experiments—we are not able to compare the negative curative effect in our paretic animals with the beneficial effect obtained by Evans and Burr in some of their mildly affected animals.

The neuropathic disturbances described above bear a stronger resemblance to the neuropathic disturbances in rats fed on a diet low in vitamin A described by Aberle [1934].

In her animals the clinical phenomena appeared successively in the following order: spreading of the hind legs when in motion increasing to pronounced straddling, ataxia, later on spasticity and finally helpless trailing of the hind quarters and hind legs when walking. Aberle did not observe disturbances of sensibility or atrophy of the hind quarters or hind legs, or even atrophic disturbances of the skin or fur. On the other hand, Aberle observed incontinency of urine in several of her animals, a phenomenon which I have not observed in mine. On the contrary, I have got the impression that many of the paretic animals have had a greatly increased capacity of the bladder. Finally, the paresis in Aberle's animals was cured by large doses of vitamin A, a result which is quite different from my experience.

I wish to emphasise that the clinical phenomena in the skin and nervous system do not resemble those seen in rats with experimental beriberi [Hofmeister, 1922; Stern and Findlay, 1929] or rat pellagra [Goldberger and Lillie, 1926; Clemmensen, 1933]. The fact that the content of yeast in the food mixture corresponds with a daily intake of 0·7 g. dried yeast speaks strongly against the supposition of a lack of vitamin B_1 or B_2 as an aetiological factor.

Similarly lack of vitamins A and D may be left out of consideration since more than sufficient daily doses of these vitamins were given. The negative therapeutic result of greater doses of cod-liver oil points also in the same direction.

The following facts make it probable that lack of vitamin E is the cause of the paresis. Vitamin E is the only definitely known vitamin that was lacking in the food mixture. If the animals in the three groups be compared, it is a striking fact that all the non-paralytic animals had an addition of vitamin E (wheat germ oil) during one or more pregnancies. None of the paretic animals was given wheat germ oil. They were given substances free from vitamin E or containing very little of it, far less than sufficient to permit of a successful pregnancy. All the paretic animals have, then, suffered for a long period of time—4–7 months—from a pronounced lack of vitamin E. The non-paretic animals were given vitamin E preparations relatively early and thereby replenished their deposits. By reason of this effect of protection against paresis by early dosing with vitamin E, in conjunction with the above-mentioned conditions, I consider that the origin of the paresis is lack of vitamin E of long standing and very high degree.

REFERENCES.

Aberle (1934). *J. Nutr.* **7**, 445.

Clemmensen (1933). *Acta Pathol. Microbiol. Scand.* **10**, 304.

Cummings and Mattill (1930). *J. Nutr.* **3**, 421.

Evans and Bishop (1922). *Anat. Rec.* **23**, *Proc.* 17.

—— and Burr (1927). *Mem. Univ. Calif. Monograph*, 8.

—— —— (1928). *J. Biol. Chem.* **76**, 273.

Fridericia (1924). *J. Biol. Chem.* **62**, 471.

Goldberger and Lillie (1926). *Publ. Health Rep. Wash.* **41**, 1025 (quoted in Vitamins (1932) *Med. Res. Council. Spec. Rep. Series*, No. 167).

Gudjonsson (1930, 1). *Dissertation. Acta Pathol. Microbiol. Scand. Suppl.* **4**, 1930.

—— (1930, 2). *Biochem. J.* **24**, 1591.

Hofmeister (1922). *Biochem. Z.* **129**, 477.

Hohlweg und Dohrn (1930). *Z. ges. exp. Med.* **71**, 762.

Mason (1933). *Amer. J. Anat.* **52**, 153.

Mattill and Crawford (1931). *Ind. Eng. Chem.* **22**, 341.

Stern and Findlay (1929). *J. Path. Bact.* **32**, 63.

Vogt-Møller (1931). *Hospitalstidende*, **74**, 567.

—— (1934). *Hospitalstidende*, **77**, 674.

XCII. THE USE OF SODIUM FLUORIDE AS A BLOOD ANTICOAGULANT IN BLOOD PHOSPHORUS DETERMINATIONS.

By JAN CORNELIS JOHAN BURKENS.

From the Laboratory of General Pathology, University of Amsterdam.

(*Received January 29th, 1935.*)

For estimating the acid-soluble phosphorus fractions of the blood—especially the inorganic blood phosphorus—we possess an excellent colorimetric technique of a simple character; in capable hands the experimental error should not be more than 3 %.

The real difficulty in blood phosphorus estimations is that the several acid-soluble fractions are extremely unstable. The organic acid-soluble compounds are rapidly hydrolysed in shed blood; when blood is allowed to stand without separating the red blood cells from the plasma a rapid and great increase of inorganic phosphorus results. But even if the red blood corpuscles are separated immediately after the withdrawal of the blood an increase in the plasma inorganic phosphorus is to be expected, as blood plasma contains an amount of ester-phosphorus corresponding to 0·2–0·5 mg. P per 100 ml. of blood; in some cases even 1·0–2·0 mg. P per 100 ml. of plasma can be found without any appreciable haemolysis. If blood becomes more alkaline ester synthesis is possible from inorganic phosphorus, resulting in a fall of the inorganic phosphorus content; this is liable to happen if blood is strongly shaken to defibrinate it: much CO_2 is lost and the blood becomes more alkaline.

The hydrolysis of ester-phosphorus takes place both in serum and in plasma of blood to which oxalate, citrate, hirudin or heparin is added as anticoagulant[1], as Table I shows.

Table I. *Inorganic blood phosphorus in mg. per 100 ml. of blood.*

	Oxalate		Citrate		Hirudin	
Immediate determination	3·26	2·86	3·29	3·20	4·10	3·13
After 1 hour	3·45	3·10	—	3·33	4·20	—
After 5 hours	—	3·28	3·73	3·60	—	3·42

Blood standing at room temperature.

It is obvious, and this fact is well known to all workers on phosphorus metabolism, that only determinations done in blood immediately after the blood has been collected have any value. In scientific work this is no great objection, but in clinical work this fact offers a great difficulty. Many physicians are unable to do an analysis immediately after the withdrawal of the blood, and in many cases it is impossible even to keep the blood at freezing temperature because it must be sent to a laboratory somewhere else. Many determinations which physicians out of town ask us to do give useless results for this reason.

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[1] Heparin is unsuitable for blood in which phosphorus must be determined as it contains phosphorus.

The impression is often held that there is no longer any risk of an undue increase of inorganic blood phosphorus if blood or plasma has been precipitated with trichloroacetic acid, and that the filtrate obtained in this way can be kept for some time, as the strong acid should prevent all phosphatase action. This is not true: the same hydrolysis will occur in the acid protein-free filtrate. Table II illustrates this fact; the figures are taken from experiments of Byrom and of myself [Byrom, 1931].

Table II. *Inorganic phosphorus in protein-free filtrate after precipitation with 20 % trichloroacetic acid (mg. per 100 ml. of blood).*

	Room temperature	38°
Byrom:		
0 hours after precipitation	4·89	4·89
5 ,,	5·06	—
24 ,,	5·62	6·50
48 ,,	5·82	7·10
Burkens:		
0 hours after precipitation	2·80	2·80
5 ,,	3·58	3·71
24 ,,	3·85	4·70

In blood, serum, plasma or protein-free filtrates, estimations give quite unreliable results after a few hours. Yet this difficulty can be avoided quite easily by using sodium fluoride to prevent the clotting of the blood instead of the ordinary anticoagulants.

Sodium fluoride has a double action on the blood: it prevents clotting when added in the same concentration as that usually employed with oxalate; and it prevents all phosphatase action. The result is that blood, when treated with sodium fluoride, can be kept for days without any hydrolysis or synthesis of ester-phosphorus occurring. The amount of inorganic phosphorus of blood or plasma remains entirely unchanged, as do the relative amounts of the several acid-soluble fractions. This is even the case if the blood is laked.[1]

10 mg. of sodium fluoride as powder were added per 5 ml. of blood.

One must be sure that the adding of sodium fluoride does not have more influence on the development of the colour in the colorimetric analysis than has oxalate. Phosphorus estimations made simultaneously in serum and in plasma of blood after adding oxalate or fluoride give identical results (Table III).

Table III. *Inorganic phosphorus in serum and plasma of blood to which oxalate or fluoride had been added (mg. per 100 ml.).*

Serum	2·92	3·65	3·08
Oxalate-plasma	2·95	3·60	3·15
Fluoride-plasma	2·90	3·60	3·15

Table IV shows that keeping the plasma for hours at 38° causes no increase in the amount of inorganic phosphorus if sodium fluoride has been added. A protein-free acid filtrate of blood can also be kept. Of course evaporation must be prevented.

After addition of sodium fluoride as a blood anticoagulant, blood or plasma can be despatched without running any risk of obtaining faulty results. If it is advisable to perform the estimation on serum, sodium fluoride can be added after the separation of the serum. Blood, plasma or serum can be kept in this way till ultimately (after several days) the growth of bacteria in some cases will cause decrease of the amount of inorganic phosphorus.

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[1] If laked blood is kept for hours at 38° ultimately ester hydrolysis occurred.

Table IV. *Inorganic blood phosphorus (mg. per 100 ml. of plasma).*

Fluoride-plasma:						
Immediate determination	3·52	3·20	2·78	2·68	4·89	3·20
After 5 hours at room temperature	3·54	3·21	—	—	—	—
After 48 hours at room temperature	—	—	—	2·73	4·97	—
After 16 hours at 38°	—	—	2·83	—	4·97	3·15
Protein-free filtrate of fluoride-plasma:						
Immediate determination	3·50	2·85	4·95	—	—	—
After 5 hours at room temperature	3·50	—	4·95	—	—	—
After 16 hours at 38°	—	2·80	4·95	—	—	—

Sodium fluoride is not very soluble in water and therefore it is impossible to prepare a concentrated (*e.g.* 20 %) solution. It is necessary to add the sodium fluoride as powder: 10 mg. per 5 ml. of blood. A little more or less makes no difference. Very large amounts of sodium fluoride prevent all development of colour in the colorimetric phosphorus analysis, exactly as is the case with high concentrations of oxalate or citrate. A 2 % solution of sodium fluoride in distilled water can also be used: 1 part to 9 parts of blood. In that case there arises some difficulty in calculating the dilution of the blood. If the analysis is performed in whole blood the dilution is 1:10 and the amount found must be multiplied by 10/9; if the estimation is made in plasma it is necessary to reckon with the fact that only the blood plasma is diluted. Actually it would be necessary in cases where a 2 % sodium fluoride solution is used instead of the powder to determine the relative volume of the plasma before and after the adding of the sodium fluoride. However, I found that if the blood had a normal or nearly normal ratio of the relative volumes of corpuscles and plasma the addition of one-tenth part of a 2 % solution of sodium fluoride results in a dilution of the plasma of 14–16 %. By multiplying the value found by 100/85 the real amount is estimated fairly accurately.

SUMMARY.

On standing an increase in inorganic blood phosphorus is found in serum or in plasma of blood to which oxalate, citrate or hirudin has been added to prevent clotting; in the acid protein-free filtrate of blood, plasma or serum the same increase occurs. Adding sodium fluoride prevents clotting of the blood and also all phosphatase action. Ester-phosphorus hydrolysis and synthesis do not occur and correct values for inorganic phosphorus are ensured, even when the blood or plasma is kept for hours or days at room temperature or at 38°. When the correct amount of sodium fluoride (10 mg. per 5 ml. of blood) is used no influence is found on the colour development in the analysis.

REFERENCE.

Byrom (1931). The clinical significance of blood phosphorus. Clinical interpretation of aids to diagnosis. (London.)

XCIII. THE BIURET METHOD OF ESTIMATING ALBUMIN AND GLOBULIN IN SERUM AND URINE.

By JOSEPH FINE.

From the Laboratory of Chemical Pathology, St Bartholomew's Hospital.

(*Received January 30th, 1935.*)

QUANTITATIVE determinations of serum and urinary proteins are of considerable clinical importance; that such estimations are made relatively infrequently is probably due to the lack of a simple yet trustworthy technique.

The methods of Esbach and Aufrecht are notoriously crude, while the gravimetric and the Kjeldahl methods are too laborious for ordinary clinical purposes. Of the colorimetric procedures the biuret method and that of Wu [1922] are both reliable, the error usually not exceeding 5 % in experienced hands. The latter however has the disadvantage that the calculations are somewhat involved, and it is moreover more lengthy than the biuret method modified as described below.

THE BIURET METHOD AS HITHERTO CARRIED OUT.

Protein is separated by salting out or by coagulation, and the precipitate is treated with NaOH and $CuSO_4$. The resulting violet colour is compared with that of a standard solution (usually of protein) similarly treated.

The method was first introduced by Riegler [1914] who applied it to urine and used as a standard pure dry serum. Autenrieth [1915] used a standard serum solution diluted with normal urine, and also extended the method successfully to the estimation of proteins in ascitic fluid and serum [1917]. Hiller [1927] proposed as standard a solution of 0·266 % pure biuret.

DISCUSSION OF THE BIURET METHOD.

1. *Choice of a standard.* In the writer's work biuret has been unsatisfactory for two reasons. Firstly, it is difficult to obtain pure biuret; a sample from a well-known firm contained about 55 % biuret, the remainder being urea and NH_4Cl. Until this was ascertained the protein results were consistently about double the correct values.

Secondly, the tint of the treated biuret solutions differs materially from that of protein solutions, leading to an increased personal error. The use of serum as standard obviates this difficulty.

When preserved with chloroform the serum-protein keeps for long periods; no change has been observed in 6 months.

As a standard the serum is diluted to contain 0·24 % protein. On keeping, a small deposit separates, but this is immaterial for the same result is obtained whether or not the deposit is resuspended.

2. *Biuret colour values of albumin and globulin.* Autenrieth [1917] states that on treatment equal quantities of albumin and globulin yield violet solutions of practically the same intensity. This was confirmed as follows. Solutions of albumin and globulin were obtained from a sample of serum by half saturating

with ammonium sulphate and filtering. The globulin deposit was dissolved in NaOH; the albumin in the filtrate was precipitated with $CCl_3.COOH$, the mixture centrifuged and the albumin redissolved in NaOH. The protein contents of the albumin and globulin solutions were determined by the Kjeldahl method.

Varying amounts of the two solutions were then taken and the total protein content in each estimated in duplicate by the biuret method (see technique below) using as standard first the albumin solution and then the globulin solution. The results are shown in Table I.

Table I.

Volume of solution estimated ml.	Protein content (Kjeldahl) mg.	Protein content, biuret method: Albumin standard mg.	% error		Globulin standard mg.	% error	
Albumin 2	10·4	10·4	0	Mean error -2·5 %	9·9	-5	Mean error -5·6 %
2·5	13·0	13·0	0		12·6	-3	
3	15·6	15·2	-3		14·7	-6	
3·5	18·2	17·3	-5		16·9	-7	
4	20·8	20·2	-3		19·4	-7	
4·5	23·4	22·5	-4		21·9	-6	
Globulin 2	7·4	8·06	+9	Mean error +4 %	7·4	+1	Mean error -1·4 %
3	11·1	10·9	-2		10·4	-6	
4	14·8	15·2	+3		14·8	0	
5	18·5	19·1	+3		18·5	0	
6	22·2	22·75	+2·5		22·2	0	

Table I shows that out of 22 estimations by the biuret method the error is between 0 and 5 % in 16 cases and between 5 and 10 % in 6 cases, and that accuracy is greatest when an albumin standard is used to estimate albumin, and a globulin standard used to estimate globulin.

When a globulin standard is used to estimate albumin the results tend to be low, and when albumin is used to estimate globulin the results tend to be high. These results suggest that globulin has a biuret colour value slightly higher than that of albumin.

The difference, however, is small and is within the range of experimental error, particularly as there was a slight difference in tint between the colorimetric solutions obtained from the albumin and globulin standards (a difference infrequently obtained in other estimations).

3. *Discrepancies between albumin results obtained by different salting-out methods.* Howe [1923] states that all the proteins of plasma except albumin are precipitated by

(1) $1{\cdot}5M$ Na_2SO_4 (21·3 %),
or (2) $2M$ $(NH_4)_2SO_4$ (26·4 % or "half saturated"),
or (3) $2{\cdot}375M$ $MgSO_4$ ("saturated").

Race [1932] points out that in Howe's own work these three methods do not give the same albumin figures and my own results confirm this.

Table II gives the percentages of albumin in various sera after salting out with Na_2SO_4 and $(NH_4)_2SO_4$ respectively and applying the biuret method.

Out of a total of 9 estimations only once has salting out with ammonium sulphate led to a lower result than with sodium sulphate; in all other cases the results with ammonium sulphate have been higher as illustrated in Table II.

Table II. *Albumin percentages of serum.*

Serum	Using 21·3 % Na_2SO_4 %	Using 26·4 % $(NH_4)_2SO_4$ %	% difference
Ox	3·80	4·40	+16
Human H.	4·40	4·75	+ 8
„ B.	4·55	5·00	+10
„ T.	4·44	5·06	+14
„ W.	4·73	5·20	+10
Mixed human	4·80	5·70	+19

Fig. 1 illustrates the amount of protein precipitated in three sera by increasing concentrations of ammonium sulphate and shows that to obtain protein precipitates equal to those brought down by 21·3 % sodium sulphate the respective concentrations of 4*M* ammonium sulphate required were 48, 59 and 65 %.

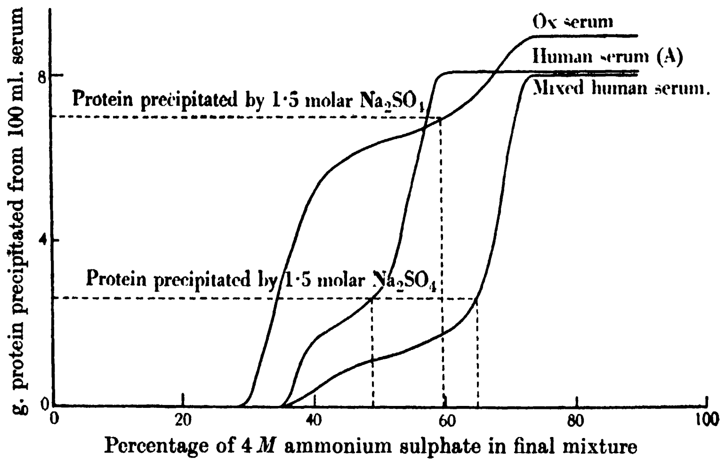

Fig. 1.

It is clear that 2*M* ammonium sulphate is not invariably equivalent to 1·5*M* sodium sulphate in fractionating proteins from serum.

If globulin is defined as that portion of the serum proteins which is precipitated by 2*M* ammonium sulphate, salting out by 1·5*M* sodium sulphate may give different results for "globulin", and further such differences are irregular.

Similarly if globulin is defined as that portion of the serum proteins which is precipitated by 1·5*M* sodium sulphate, salting out by 2*M* ammonium sulphate will often give different results.

It is important that this discrepancy should be generally realised, and that some convention should be adopted in defining "globulin". In the meanwhile in the writer's work ammonium sulphate has been employed, because it avoids the need for working at 37° which is essential for salting out by sodium sulphate in Howe's technique.

4. *Time required for the method.* Any accurate method of protein estimation involves many hours, and even the colorimetric methods are greatly delayed in estimating albumin owing to the customary procedure of allowing the Na_2SO_4-protein mixture to stand for 3 hours before filtering. I have found that this filtration can be completed in 15 minutes without affecting the result if

the salt + protein is filtered immediately after mixing and refiltered as soon as the filtrate comes through clear.

Performed in this way the estimation of albumin and globulin can be completed in less than 45 minutes.

TECHNIQUE OF MODIFIED METHOD FOR SERUM PROTEINS.

1. *Total proteins.* With a blood pipette calibrated "to contain", measure 0·2 ml. serum into 2 or 3 ml. distilled water in a 15 ml. graduated centrifuge-tube: add more distilled water up to 5 ml. and 10 % $CCl_3.COOH$ up to 10 ml.: mix, centrifuge for 3 minutes at about 3000 revolutions per minute.

Decant the clear supernatant fluid: add 1 ml. 30 % NaOH and about 3 ml. distilled water and shake: when the protein has dissolved add more water up to 9 ml. then 5 % $CuSO_4$ up to 10 ml. Shake for 10 seconds.[1] Centrifuge as before. Decant the supernatant fluid for comparison in the colorimeter with the standard.

2. *Albumin.* With an Ostwald pipette calibrated to "blow out", measure 0·5 ml. serum into a test-tube and add 9·5 ml. of 27·79 % ammonium sulphate (the resulting mixture is 2 *M*). Mix, filter through No. 44 Whatman filter-paper, and when the filtrate comes through clear refilter through the same paper. Measure 5 ml. of the filtrate into a centrifuge-tube and add 5 ml. 10 % trichloroacetic acid. Continue as for total protein.

3. *Preparation of the standard.* Measure 5 ml. of the standard 0·24 % solution of protein into a centrifuge-tube, add 5 ml. 10 % $CCl_3.COOH$ and continue as for total protein.

Calculation. If the unknown is set at 20 mm. and the readings of the standard for total protein and albumin are X and Y mm. respectively, then

$$\begin{aligned}\text{Total protein} &= 0{\cdot}3X\ \%.\\ \text{Albumin} &= 0{\cdot}24Y\ \%.\\ \text{Globulin} &= (0{\cdot}3X - 0{\cdot}24Y)\ \%.\end{aligned}$$

Application of method to urine.

The following modifications are involved when the method is applied to urine:

1. The urine must be made faintly alkaline to litmus (about p_H 7·4).

2. The amount of urine for the determinations must vary with the total protein content as approximately determined (*e.g.* by Aufrecht's method).

When the protein content is in the region of 1 % (a figure not often exceeded in pathological urines), 1 ml. should be used for total protein, 3 ml. for albumin. When the protein content is low (about 0·25 %), the quantities used are 4 ml. and 12 ml. respectively.

At intermediate values corresponding quantities of urine are used.

3. Saturated ammonium sulphate (52·8 %) should be used for the albumin estimation, the volume added being equal to that of the urine. Half the filtrate may then be used as in the case of the serum method.

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[1] Autenrieth states that shaking for 3 minutes is necessary for the maximum development of colour, but I have found no significant difference between shaking 5, 10, 30 and 180 seconds.

SUMMARY.

1. A modification is described of the biuret method of estimating serum proteins.

2. It is confirmed that the violet colour developed by equal amounts of albumin and globulin is practically the same, and that therefore serum may be used as the standard solution for estimating both total protein and albumin.

3. The standard advocated is serum diluted to contain 0·24 % protein. It keeps for several months at least in the presence of chloroform.

4. Ammonium sulphate ($2M$ finally) is used for the separation of globulin. The results of the albumin estimation do not correspond with those obtained when sodium sulphate ($1{\cdot}5M$) is employed and are usually higher. This is important as it has been claimed that fractionation of the protein is the same with both.

5. The modifications required for urine are described.

I am indebted to Dr G. A. Harrison for his interest in this work and for the facilities provided in his laboratory.

REFERENCES.

Autenrieth (1915). *Munch. med. Woch.* **62**, 1418.
—— (1917). *Munch. med. Woch.* **64**, 241.
Hiller (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 385.
Howe (1923). *J. Biol. Chem.* **57**, 241.
Race (1932). *Biochem. J.* **26**, 1573.
Riegler (1914). *Z. anal. Chem.* **53**, 242.
Wu (1922). *J. Biol. Chem.* **51**, 33.

XCIV. ON THE CHANGES OF WHEAT PROTEINS UNDER THE ACTION OF FLOUR AND YEAST ENZYMES.

By ANDREY VASSILIEVICH BLAGOVESCHENSKI
AND MARIA PETROVNA YURGENSON.

Scientific Research Institute of Bread Making, Moscow, U.S.S.R.

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THE observation of Blagoveschenski and Sossiedov [1933] of the occurrence in wheat flour of an enzyme with the specific power of disaggregating gluten made it desirable to study in greater detail the changes undergone by wheat proteins in the process of bread making.

In a preliminary orientating experiment an examination was made of the distribution of nitrogen in the flour itself and in the mixture at various stages up to baking; the wheat proteins were separated into fractions by successive extractions with (1) water, 50 parts, (2) 72 % alcohol, 30 parts, (3) 10 % NaCl, 30 parts and (4) 2 % NaOH, 50 parts; these fractions contained respectively (1) leucosin and non-protein nitrogen, (2) gliadin, (3) globulin, (4) glutenin. In the water-soluble fraction additional determinations were made of the nitrogen precipitable with lead acetate and of the free amino-nitrogen by the method of Linderström-Lang [1928].

The results obtained in this manner from two samples of flour are shown in Table I from which it appears that during fermentation of the dough there is a marked decrease in the water-soluble nitrogen and an increase in the gliadin-nitrogen. The glutenin remains constant as does also the total nitrogen if allowance be made for the slight increase of the latter due to addition of flour during making. The completeness of precipitation of the water-soluble nitrogen by lead acetate indicates the absence of any appreciable degree of proteolysis except in the sponge before making, and any increase which occurs in this fraction is therefore to be ascribed to increased solubility of the proteins.

Table I. *Distribution of nitrogen in the different fractions.*

mg. N per g. flour.

	Flour No. 1					Flour No. 2				
		Sponge		Dough			Sponge		Dough	
	Flour	Before making	At making	After mixing	Before baking	Flour	Before making	At making	After mixing	Before baking
Nitrogen of water-soluble fraction	4·37	5·95	8·23	8·57	4·50	3·05	4·85	6·17	5·81	3·43
Nitrogen in the lead precipitate	4·25	5·09	8·26	8·38	4·50	3·00	4·30	6·08	5·80	3·46
Nitrogen of free amino-groups of water-soluble fraction	0·26	0·62	0·56	0·84	0·84	0·25	0·50	0·30	0·67	0·78
Gliadin	6·37	5·15	5·10	5·02	7·90	5·04	4·18	4·21	4·20	6·22
Globulin	3·86	3·62	2·26	3·57	3·55	3·50	3·62	2·09	3·23	3·15
Glutenin	11·59	11·43	11·43	11·43	11·43	9·21	8·18	8·18	8·18	8·18
Total nitrogen	25·11	26·07	26·46	27·53	26·46	19·8	20·73	20·65	21·29	20·98

Action of flour enzymes on wheat proteins.

Preparations of flour enzymes were made by extracting 10 g. of flour with 100 ml. of $M/6$ citrate-phosphate buffer at varying p_H and 37° for 24 hours; the filtered extracts were mixed with equal volumes of glycerol. For the experiments 1 g. of freshly prepared wheat proteins was mixed with 20 ml. of buffer solution, 5 ml. of enzyme extract and 1 ml. of toluene; fractional analyses of the products were made after digestion for 48 hours at 37°. The results of these analyses are shown in Table II, Experiment (A).

Table II. *The action of flour proteinases upon wheat proteins.*

mg. N per g. wheat proteins.

	p_H 5·1				p_H 4·8				p_H 3·8			
	Water sol. fract.	Gliadin	Globulin	Glutenin	Water sol. fract.	Gliadin	Globulin	Glutenin	Water sol. fract.	Gliadin	Globulin	Glutenin
Experiment (A)	22·2	15·6	12·8	51·5	22·4	15·6	12·7	51·0	20·1	15·8	14·9	51·6
Fresh enzyme (a)	2·4	2·4	2·4	2·4	2·4	2·4	2·4	2·4	2·4	2·4	2·4	2·4
Fresh gluten (b)	16·1	16·4	14·3	51·2	16·0	16·0	14·3	51·0	15·8	16·4	14·5	51·2
Difference: $A-(a+b)$	+3·7	−3·2	−1·5	−2·1	+4·0	−2·8	−4·0	−2·4	+1·9	−4·0	−2·0	−2·0
Enzyme and gluten both heated	18·3	16·5	14·6	51·5	18·1	16·3	14·5	51·04	18·0	16·2	14·4	51·1
Heated enzyme and fresh gluten	18·6	16·7	14·8	51·4	18·4	16·4	14·7	51·1	18·2	16·2	14·5	51·1
Fresh enzyme and heated gluten	18·6	16·5	14·4	51·2	18·1	16·3	14·4	51·1	17·9	16·3	14·5	51·2

The possibility of adsorption of proteinases by the wheat protein preparation has to be considered. An attempt to eliminate this factor in the usual way by heating the preparation failed, since, as is shown by the figures in the second, third and fourth lines of Table II, heating rendered the proteins resistant to the action of the flour enzymes. Determinations were therefore made of the changes occurring in the protein and enzyme preparations separately on digestion under the above conditions (a and b in Table II); the differences between the figures obtained in the original experiment (A) and the sum of those obtained in experiments (a) and (b) were taken to represent the true action of the flour enzymes. It is evident that there is an increase in the water-soluble nitrogen and a decrease in the various protein fractions, this decrease being most marked with gliadin and least with glutenin.

Effect of p_H. The effect of p_H on the action of the flour enzymes on the wheat proteins is shown in Table III. The experimental mixtures consisted of 1 g. wheat proteins, 20 ml. buffer and 5 ml. enzyme extract; in the controls 5 ml. of buffer were substituted for the enzyme. It is seen that the maximum increase in water-soluble nitrogen occurs at p_H 8·5 and the minimum at p_H 7·5; furthermore these reactions correspond with the maxima and minima of the decreases in the various protein fractions.

It is noteworthy that glutenin remains unaffected during the first 24 hours, suggesting that it may be protected by the other proteins so long as these remain intact.

Changes in the amino-nitrogen. Table IV gives the results of amino-nitrogen determinations in the various fractions during digestion of wheat proteins with flour enzymes. Increases occur in both experimental and control solutions but the differences between the experiments and their corresponding controls are significant only in the case of the water-soluble fraction, and here only at the

Table III. *Effect of action of flour enzymes on wheat proteins.*

mg. N per g. wheat proteins.

	24 hours		48 hours		72 hours		144 hours		Increase or decrease in 144 hours
p_H	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	
				Water-soluble fraction.					
3·8	20·1	18·4	36·6	19·0	43·8	19·9	59·4	20·0	+39·4
4·0	21·8	18·4	38·5	19·0	45·2	20·0	58·8	19·9	+38·9
4·8	22·4	18·4	36·2	19·0	45·5	19·9	58·8	19·9	+38·9
5·1	22·2	18·6	38·0	18·7	45·4	19·9	58·7	19·9	+38·8
6·5	23·4	18·1	—	—	—	—	—	—	—
7·5	19·8	18·1	29·0	20·0	39·1	20·0	43·7	19·9	+23·8
8·5	27·2	19·0	47·8	20·3	67·3	20·4	81·0	20·4	+60·6
				Gliadin.					
3·8	15·8	16·4	14·8	16·6	10·9	15·5	5·6	15·6	−10·0
4·0	15·9	16·4	14·0	16·7	9·8	15·4	5·9	15·5	−9·6
4·8	15·6	16·4	14·9	16·7	10·0	15·5	6·0	15·5	−9·5
5·1	14·6	16·7	14·5	16·7	10·0	15·6	6·0	15·6	−9·5
6·5	16·3	20·7	—	—	—	—	—	—	—
7·5	18·9	20·7	16·1	17·9	12·1	17·9	11·1	15·6	−4·5
8·5	15·5	20·7	11·4	17·9	5·5	16·9	3·6	16·9	−13·3
				Globulin.					
3·8	13·9	14·5	7·3	14·8	6·4	14·7	2·3	14·7	−12·4
4·0	13·0	14·7	6·5	14·8	6·0	14·7	4·0	14·7	−10·7
4·8	12·7	14·7	7·5	14·8	6·2	14·7	3·4	14·7	−11·3
5·1	12·8	14·8	7·1	14·8	6·2	14·7	3·4	14·7	−11·3
6·5	9·4	10·5	—	—	—	—	—	—	—
7·5	11·4	10·5	7·3	11·6	5·9	11·6	4·8	14·6	−9·8
8·5	7·7	10·4	6·5	12·6	4·5	13·1	2·6	13·2	−10·0
				Glutenin.					
3·8	51·62	51·1	42·4	51·4	40·1	51·4	34·1	51·4	−16·3
4·0	51·12	51·1	42·6	51·5	40·1	51·4	31·9	51·4	−19·5
4·8	51·0	51·1	42·5	51·4	40·1	50·1	31·9	51·3	−19·4
5·1	51·5	51·5	42·6	51·6	40·1	50·1	31·9	51·3	−19·4
6·5	51·0	51·0	—	—	—	—	—	—	—
7·5	51·0	51·1	48·7	51·8	45·0	51·8	42·4	51·3	−8·9
8·5	51·0	51·1	36·6	51·6	26·7	50·6	18·1	50·5	−32·4

more acid reactions; at p_H 8·5 where the maximum solution of proteins occurs the changes in amino-nitrogen are negligible. It is clear therefore that, except at the acid reactions (where some hydrolysis of the dissolved proteins must be assumed), the increased solubility of the proteins which has been shown to occur under the influence of the flour enzymes is not accompanied by true proteolysis.

Action of yeast enzymes on wheat proteins.

During the process of bread making the wheat proteins are exposed to the action of yeast enzymes as well as to that of the flour enzymes already considered. Table V gives the results of the action on wheat proteins under conditions similar to those employed in the experiments described above, of preparations made by extracting 10 g. of fresh pressed yeast with 100 ml. of buffer in presence of toluene at 37° for 24 hours, the filtered extract being mixed with an equal volume of glycerol. It will be observed that in contrast with the flour enzymes the yeast enzymes have no action at p_H 8·5; moreover the figures for amino-nitrogen in Table VI indicate that at the point of maximum activity (p_H 4·8–6·5) the action of the yeast enzymes consists of a true proteolysis.

Table IV. *The nitrogen of free amino-groups.*

p_H	24 hours		48 hours		72 hours		144 hours		Difference at 144 hours
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	
(*a*) Water-soluble fraction.									
3·8	2·75	6·3	5·4	18·3	8·2	27·0	10·0	29·0	+19·0
4·0	2·4	6·7	5·1	18·7	8·1	27·2	10·1	29·2	+19·1
4·8	2·4	5·9	5·2	14·4	8·0	25·6	9·8	26·8	+17·0
5·1	2·4	5·9	4·9	12·6	8·1	24·1	9·8	25·8	+16·0
7·5	2·4	2·5	2·5	4·0	2·6	5·3	5·3	8·2	+2·9
8·5	2·45	3·5	3·6	4·3	8·5	8·7	15·0	15·9	+0·9
(*b*) Gliadin fraction.									
3·8	4·7	2·8	4·0	1·0	3·7	0·4	0·2	0·2	0
4·0	4·5	2·8	4·0	1·0	3·6	3·6	2·0	0·2	−1·8
4·8	4·4	3·1	4·0	1·1	3·6	3·6	2·6	0·4	−2·2
5·1	4·4	3·1	4·0	1·2	3·6	3·6	4·2	3·5	−0·7
7·5	4·5	4·5	4·4	4·4	4·5	4·5	4·2	3·5	−0·7
8·5	4·5	4·6	4·4	4·5	3·5	3·5	2·0	2·0	0
(*c*) Globulin fraction.									
3·8	4·6	2·8	4·0	1·1	3·7	0·4	0·2	0·2	0
4·0	4·5	2·9	4·0	1·1	3·6	0·5	2·0	0·2	−1·5
4·8	4·5	3·2	4·0	1·1	3·5	0·7	2·6	0·4	−2·2
5·1	4·5	3·2	4·1	1·2	3·6	0·8	2·7	0·4	−2·3
7·5	4·5	4·5	4·4	4·4	4·5	4·1	4·2	3·5	−0·7
8·5	4·5	4·9	4·4	4·7	3·4	3·5	1·5	1·5	0
(*d*) Glutenin fraction.									
3·8	5·2	3·8	4·8	2·2	2·2	1·2	2·2	0·6	−1·6
4·0	5·1	3·8	4·7	2·3	2·3	2·0	2·2	0·6	−1·6
4·8	4·8	3·9	4·7	2·4	2·2	2·0	2·3	0·6	−1·7
5·1	4·8	4·0	4·7	2·4	2·3	2·1	2·3	0·7	−1·6
7·5	5·1	5·1	5·1	5·1	5·1	5·0	5·1	4·3	−0·8
8·5	5·1	5·1	4·9	4·9	3·3	3·3	1·4	1·4	0

Table V. *The effect of yeast ferments on the total nitrogen of various fractions.*

mg. N per g. wheat proteins.

p_H	24 hours		48 hours		72 hours		144 hours		Difference at 144 hours
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	
(*a*) Water-soluble fraction.									
4·0	18·4	25·3	18·9	39·2	19·9	43·9	19·9	55·2	+35·3
4·8	18·4	36·9	19·0	47·2	19·9	54·0	19·9	72·5	+52·6
6·5	18·0	33·6	18·7	42·9	18·6	50·8	18·7	71·7	+53·0
8·5	19·0	19·1	20·3	20·4	20·4	20·4	20·4	20·5	+0·1
(*b*) Gliadin fraction.									
4·0	16·3	13·1	16·7	7·1	15·4	6·6	15·5	4·7	−10·8
4·8	16·4	8·6	16·7	4·1	16·5	3·0	15·5	2·2	−13·3
6·5	20·8	10·2	20·4	5·5	20·4	4·4	20·4	2·3	−18·1
8·5	20·6	20·7	17·9	17·9	16·9	17·9	16·9	16·7	−0·2
(*c*) Globulin fraction.									
4·0	14·7	12·2	14·8	6·8	14·7	5·3	14·7	4·2	−10·5
4·8	14·7	6·1	14·8	2·5	14·7	1·1	14·7	1·1	−13·6
6·5	10·6	7·7	10·4	5·3	10·4	4·1	10·4	2·1	−8·3
8·5	10·4	10·4	12·5	12·5	13·1	13·5	13·2	13·2	0
(*d*) Glutenin fraction.									
4·0	50·9	50·0	51·5	49·7	50·4	45·6	50·4	37·8	−12·6
4·8	51·1	50·1	50·0	47·7	50·1	43·5	51·3	24·9	−26·4
6·5	50·1	50·0	50·6	49·1	50·6	44·4	50·1	25·8	−24·3
8·5	51·1	51·1	51·5	51·5	50·5	50·5	50·5	50·5	0

Table VI. *Effect of yeast proteases upon the free amino-nitrogen of the different fractions of wheat proteins.*

mg. N per g. wheat proteins.

p_H	24 hours		48 hours		72 hours		144 hours		Difference at 144 hours
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	
				(*a*) Water-soluble fraction.					
4·0	2·4	10·5	5·1	18·3	8·1	27·2	10·0	29·2	+19·2
4·8	2·0	18·7	2·4	27·5	5·1	40·7	10·1	51·1	+41·0
6·5	2·5	15·7	2·5	24·9	5·6	39·2	9·7	48·5	+38·8
8·5	2·5	3·5	3·6	4·3	8·5	8·7	15·9	15·8	−0·1
				(*b*) Gliadin fraction.					
4·0	4·5	5·7	4·0	3·8	3·6	2·3	2·0	2·0	+1·8
4·8	3·6	4·2	4·4	3·9	4·0	2·5	2·0	2·0	0
6·5	4·5	5·3	4·5	5·0	4·0	4·4	2·6	2·0	−0·6
8·5	4·5	4·5	4·4	4·5	3·5	3·5	2·0	2·0	0
				(*c*) Globulin fraction.					
4·0	4·5	5·3	4·0	3·0	3·6	2·5	2·0	2·8	+0·8
4·8	3·5	3·6	4·5	2·1	4·0	1·1	2·0	1·0	−1·0
6·5	4·5	4·5	4·5	5·4	3·7	4·0	2·7	2·0	−0·7
8·5	4·8	4·8	4·4	4·7	3·4	3·5	1·5	1·5	0
				(*d*) Glutenin fraction.					
4·0	5·1	5·8	4·5	8·0	2·3	10·1	2·2	11·4	+9·2
4·8	5·0	5·8	4·8	8·9	4·7	11·2	2·2	12·0	+9·8
6·5	5·0	6·1	4·9	8·0	4·8	10·3	2·5	12·7	+10·2
8·5	5·1	5·1	4·9	4·9	3·3	3·3	1·4	1·4	0

The effects of temperature on the activities of the flour and yeast enzymes are shown in Table VII from which it will be seen that whilst the flour enzymes exhibit an optimum at 37–40° the yeast enzymes are equally active over the range 20–37° and only show a falling off when a temperature of 45° is reached.

Table VII. *The effect of temperature upon the action of the proteolytic ferments of flour (A) and yeast (B) upon wheat proteins during 24 hours at* p_H *4·8.*

mg. N per g. wheat proteins.

	20°			37°			40°			45°		
A.	Beginning	End	Difference	Beginning	End	Difference	Beginning	End	Difference	Beginning	End	Difference
Water-soluble fraction	18·4	31·9	+13·5	18·98	36·24	+17·26	18·99	36·25	+17·26	19·5	31·2	+11·7
Gliadin	17·19	15·13	−2·06	16·59	14·9	−1·8	16·71	14·93	−1·78	16·68	15·43	−1·25
Globulin	14·8	9·50	−5·3	14·8	7·5	−7·3	14·8	7·45	−7·35	14·8	9·55	−5·25
Glutenin	51·4	44·5	−6·9	51·4	42·5	−8·9	51·4	42·5	−8·9	51·32	44·9	−6·42

B.	20°			30°			37°			45°		
Water-soluble fraction	18·4	36·9	+18·5	18·4	36·9	+18·5	18·4	37·0	+18·6	18·4	28·8	+10·4
Gliadin	16·4	8·6	−7·8	16·4	8·6	−7·8	16·4	8·6	−7·8	16·4	12·5	−3·9
Globulin	14·7	6·1	−8·6	14·7	6·2	−8·6	14·7	6·2	−8·5	14·7	12·1	−2·6
Glutenin	51·1	50·1	− 1·0	51·1	50·0	−1·1	51·1	50·0	−1·1	51·0	50·0	−1·0

SUMMARY.

1. Enzymes are present in flour which exercise a definite solvent effect on wheat proteins; this effect is to be regarded as disaggregation rather than proteolysis since it is not accompanied by increase in amino-nitrogen.

2. The effect of the flour enzymes is most marked at p_H 8·5. At this p_H yeast enzymes have no action on wheat proteins. At p_H 4·8–6·5 yeast enzymes also exercise a solvent effect on wheat proteins, but this is due to a true proteolysis.

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REFERENCES.

Blagoveschenski and Sossiedov (1933). *Biochem. J.* **28**, 1575.
Linderström-Lang (1928). *Z. physiol. Chem.* **173**, 32.

XCV. RÔLE OF GLUTATHIONE IN ANAEROBIC TISSUE GLYCOLYSIS.

By ALEXANDER GEIGER.

From the Hebrew University, Jerusalem.

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The relative amounts of lactic acid produced anaerobically by animal tissues from glycogen and from glucose depend on the tissue. Skeletal muscles, while able to form a large quantity of lactic acid from glycogen, produce only a relatively small amount of lactic acid from glucose. On the other hand involuntary muscles, brain tissue, heart muscle and lungs produce far more lactic acid from glucose than from glycogen. Other tissues, such as kidney, spleen *etc.*, are able to glycolyse glycogen and glucose in about equal amount (for literature see Haarmann [1932]).

Recent evidence seems to suggest that the course of breakdown of glucose is different from that of glycogen. Thus while the presence of inorganic phosphate is essential in muscle glycolysis where glycogen is the main substrate, according to Ashford and Holmes [1929] no inorganic phosphate is necessary for lactic acid production from glucose by brain tissue. It has been later shown by Ashford [1933] that the amount of lactic acid formed by brain tissue from glycogen and from glucose when both are present is equal to the sum of the amounts formed from each substrate separately. Bumm and Fehrenbach [1930; 1931], examining the effect of different activators on the glycolytic action of different organs, suggest two ways for the breakdown of glucose one of which does not pass through the phosphoric ester stage.

There are different opinions as to the precursor of lactic acid. Neuberg and his school advocate methylglyoxal as an intermediary product. On the other side Embden and his co-workers [1933] and Meyerhof and Kiessling [1933] found that in muscle pyruvic acid is to be considered as the precursor of lactic acid. Case and Cook [1931] succeeded in isolating pyruvic acid as well as methylglyoxal from incubated muscle tissue. The quantity of pyruvic acid was always much larger than that of methylglyoxal.

In the present experiments on the rôle of glutathione in glycolysis evidence is offered in favour of the theory that the mode of anaerobic breakdown of glucose is different from that of glycogen, and that methylglyoxal can be considered as an intermediary product of glucose breakdown but not of glycogen breakdown.

METHODS.

The animals used for these experiments were albino rats from a uniformly bred strain. The animals were fed for generations on a standard diet consisting of

Caseinogen ("Physiological" Glaxo)	21 % of the total Calories
Starch	57 %
Sucrose	8 %
Olive oil	14 %
Osborne and Mendel salt mixture...	40 g. to each kg. food

In addition each rat received daily 0·5 g. dried yeast; 0·1 ml. cod-liver oil and a few drops of lemon juice. Litter-mates were used for comparative experiments.

The rats were killed by stunning or decapitation. The required organs were immediately removed, weighed and quickly ground over ice in a mortar. From the finely ground organs a fairly uniform emulsion was obtained by adding slowly during grinding an ice-cooled buffer solution or a saline solution of known volume and composition. Measured quantities of these emulsions were distributed in small Erlenmeyer flasks. From finely cut skeletal muscles weighed quantities were distributed in the Erlenmeyer flasks. For the anaerobic experiments the bottles were evacuated repeatedly and filled up with nitrogen. The incubating vessels were ordinary 50 ml. Erlenmeyer flasks each provided with a rubber stopper in which a stopcock was inserted. During the experiments these bottles were slowly rocked in a water-thermostat regulated to 37° (±1°).

The determination of lactic acid was carried out chemically by the method of Friedemann *et al.* [1927]. The original method with permanganate oxidation was used in those experiments in which methylglyoxal was the substrate. The error due to methylglyoxal was very low when permanganate was used as oxidising agent. When no methylglyoxal was present the more recent modification of this method was used with MnO_2. In the presence of methylglyoxal the trichloroacetic acid filtrates were used directly for lactic acid estimation. In the absence of methylglyoxal a copper-calcium precipitation of the neutralised trichloroacetic filtrates was carried out. Both methods gave uniform and reliable results.

The estimation of glutathione was carried out by Kuehnau's method [1931]. Qualitative nitroprusside tests were carried out according to Hopkins.

The methylglyoxal was estimated in the trichloroacetic acid filtrates by the method of Barrenscheen and Dreguss [1931]. This method was slightly modified in so far as Schott's sintered glass filters were used instead of centrifuging tubes. A weighed amount of repeatedly recrystallised methylglyoxalbis-2:4-dinitrophenylhydrazone, prepared by precipitating a methylglyoxal solution with 2:4-dinitrophenylhydrazine and dissolving a weighed amount of the recrystallised precipitate in nitrobenzene, served as standard for the colorimetric estimation of methylglyoxal. (The author is indebted to Dr M. Weizmann for the preparation of the 2:4-dinitrophenylhydrazine.) The volume of the nitrobenzene was adjusted so that 2 ml. of the solution corresponded to 0·1 mg. of methylglyoxal. 2 ml. of this solution made up with alcoholic potash solution to 100 ml. served as a standard. The nitrobenzene solution keeps very well and saves labour in preparing fresh standard solution for each experiment.

The methylglyoxal was prepared according to Neuberg and Hoffmann [1930]. Another solution of methylglyoxal was obtained by distilling dihydroxyacetone with sulphuric acid according to Neuberg. In the experiments both preparations gave the same result although both contained small quantities of impurities.

Adenosinetriphosphoric acid was prepared from rabbit muscle according to Barrenscheen and Filz [1932]. Two such preparations were used for the experiments.

Three glutathione preparations were used, one of which was a B.D.H. preparation, the other two were prepared from baker's yeast according to Pirie's method [1930]. All three preparations gave identical results. Oxidised glutathione was prepared by dissolving a weighed amount of glutathione in a known volume of 0·5 % $NaHCO_3$ solution and shaking this solution in the presence of oxygen until the disappearance of the nitroprusside reaction.

The general procedure (if not stated otherwise) was as follows. For each experiment at least four bottles were prepared, containing the buffer solution, the substrate and the other subtances. Equal volumes of the tissue emulsion were measured into each bottle, after which the bottles were quickly evacuated repeatedly, filled up with nitrogen and put in the rocking arm of the thermostat. The time was noted when the tissue emulsion was placed in the bottles. The first two bottles contained no substrate besides that contained in the tissue suspension. After adding the tissue suspension the whole contents of one bottle were immediately precipitated by trichloroacetic acid. The contents of the second bottle were precipitated at the end of the experiment. To the third and fourth bottles a known amount of the substrate was added. The contents of one of these were immediately precipitated and the contents of the second at the end of the experiment. When the influence of an activator was tested more bottles were added to the set. The final volume, the p_H and the osmotic pressure were the same in all bottles. By this arrangement the influence of interfering substances contained in the substrates and in the tissue emulsions could be excluded from the results. By subtracting the amount of lactic acid found in the first bottle from that in the second, the lactic acid production without substrate was obtained (A); by subtracting the value of the third from that of the fourth bottle the lactic acid production in the presence of the substrate was obtained (B); the amount of lactic acid produced from the added substrate alone could be calculated by subtracting from the value (B) the value (A).

COENZYMIC FUNCTION OF REDUCED GLUTATHIONE IN THE PROCESS OF LACTIC ACID FORMATION FROM GLUCOSE.

Lohmann demonstrated [1932] that muscle extracts freed completely from reduced glutathione by dialysis retained their ability to form lactic acid from glycogen but were unable to transform methylglyoxal into lactic acid. The addition of reduced glutathione restored the ability of these extracts to form lactic acid from methylglyoxal. From his experiments Lohmann concluded that reduced glutathione was the coenzyme of the methylglyoxalase. In order to explain the fact that glutathione was not required for the process of lactic acid formation from glycogen this author assumed the mechanism of conversion of synthetic methylglyoxal into lactic acid to be different from that of the methylglyoxal, formed according to the theory of Neuberg as an intermediary product of carbohydrate breakdown.

The experiments of Lohmann may, however, also suggest that methylglyoxal does not occur at all as an intermediary product in the process of glycogen breakdown in muscle. From the work of Embden *et al.* [1933] and of Meyerhof and Kiessling [1933] the intermediary formation of methylglyoxal from glycogen in muscle is very doubtful.

On the other hand methylglyoxal may be an intermediary product in the process of lactic acid formation from glucose. In this case reduced glutathione, being a coenzyme of methylglyoxalase, would have a coenzymic function in the process of glucose breakdown.

In order to test this hypothesis we first applied Lohmann's experiments to glucose by using such types of tissues as readily metabolise glucose, *i.e.* brain tissue and involuntary muscles.

(*a*) *Experiments with tissue extracts.*

The tissue extracts used in these experiments were prepared according to Lohmann, finely cut tissue being macerated in 0·5 % KCl solution. The centrifuged extracts were dialysed against 0·5 % KCl solutions through collodion membranes for 8–12 hours in ice-cooled vessels. At the end of the dialysis glutathione could still be detected in all the extracts. The extracts were therefore made slightly alkaline with $NaHCO_3$ (p_H 7·8) and were agitated for one hour with oxygen at room temperature. At the end of this period no more glutathione could be detected. For each substrate 5–7 bottles were set up provided with stopcocks as stated above. Each bottle contained 14 ml. of fluid, containing equal volumes of tissue extract. $MgCl_2$ was added in a final concentration of 8 mg./100 ml. The initial concentration of glucose or of glycogen or of methylglyoxal was 0·3 %. Phosphate buffer of p_H 7 was used in a final concentration of *M*/15. The concentration of reduced glutathione was 30 mg./100 ml. Adenosinetriphosphoric acid was added in a final concentration corresponding to 5 mg. P_2O_5 per 100 ml. The incubation lasted usually for 2 hours at 37°.

The tissue extracts had different concentrations according to the tissue. The proportions of tissue to 0·5 % KCl were as follows: 1 part muscle to 2 parts of KCl solution; 1 part brain to 5 parts of KCl solution; 1 part heart to 6 parts of KCl solution; 1 part uterus to 7 parts of KCl solution; 3 ml. of muscle extract or 4 ml. of the other extracts were used for each experiment. The results are summarised in Table I.

Table I.

		mg. lactic acid formed from the added substrate by 100 ml. of the tissue extract		
Tissue extract	Substrate	Without activator	With adenosine-triphosphoric acid	With adenosine-triphosphoric acid and glutathione
Muscle, rabbit	Glycogen	—	+89·8	+ 80·4
	Methylglyoxal	+4·96	+ 2·80	+407·0
	Glucose	-0·66	- 0·84	+ 9·9
Muscle, rat	Glycogen	—	+231·0	+207·0
	Methylglyoxal	+7·3	—	+379·0
	Glucose	+0·12	- 0·31	+ 21·3
Brain, rat	Glucose	-1·33	- 1·33	+ 20·0
	Methylglyoxal	—	+ 0·81	+142·4
,,	Glucose	-4·5	- 2·14	+ 18·9
	Methylglyoxal	+0·30	—	+ 91·0
,,	Glucose	—	+ 0·1	+ 17·4
	Methylglyoxal	—	+ 3·3	+102·0
Liver, rat	Glucose	-9·0	- 3·0	+107·0
Heart, rat	,,	+0·09	+ 0·14	+ 28·0
,,	,,	-0·8	- 1·0	+ 20·0
Uterus, rabbit	,,	+0·35	+ 3·5	+ 12·9

The experiments reported in Table I demonstrate uniformly the inability of all the tissue extracts to form lactic acid from glucose or from methylglyoxal if no reduced glutathione is present, and the ability of the same tissue extracts to produce lactic acid from glycogen even in the absence of reduced glutathione. The addition of reduced glutathione to these extracts enables them to form lactic acid from glucose and from methylglyoxal, but it does not increase the amount of lactic acid formation from glycogen. Reduced glutathione seems, according to these experiments, to be a coenzyme common to the glycolytic enzyme system and to the methylglyoxalase, but it is not concerned in the process of lactic acid formation from glycogen.

According to these experiments methylglyoxal may be an intermediary product in the formation of lactic acid from glucose, reduced glutathione being essential in both processes. It is, however, impossible to consider this methylglyoxal as an intermediary product in the breakdown of glycogen as this last process takes place even in the absence of reduced glutathione.

It is evident that in this experiment the lactic acid production is very slight. The inhibiting factor of the lactic acid production in these extracts is probably the oxidised glutathione. Since it was not possible to eliminate completely the glutathione from the tissue extract by dialysis we oxidised it. Thus all the extracts contained small quantities of oxidised glutathione. As will be shown later small quantities of oxidised glutathione are able to exert a strong inhibiting action on glycolysis.

(*b*) *Experiments with chopped tissues.*

The following experiments were made on fresh chopped tissue suspended in phosphate buffer solution. It was intended to investigate the activating effect of reduced glutathione on lactic acid production from glucose and from glycogen. As the tissue suspensions already contain preformed glutathione only the activating effect of added reduced glutathione could be investigated.

The technical details of these experiments have been described previously. Three to four rats were used for each experiment with brain tissue.

The bottles were set up as follows:

Phosphate buffer p_H 7·0	5·0 ml.
3 % solution of glucose or glycogen	1·0 ml.
Tissue emulsion, containing 0·4 g. of tissue	3·0 ml.
Adenosinetriphosphoric acid (or water)	0·5 ml. containing 0·5 mg. P_2O_5
Glutathione solution (or water) ...	0·5 ml. containing 4·0 mg. of glutathione

Adenosinetriphosphoric acid or glutathione or both were added only where it is stated in the Table. The experiments are reported in Table II.

All the experiments reported in Table II demonstrate that reduced glutathione strongly accelerates the rate of lactic acid formation from glucose but depresses to a small extent the rate of lactic acid formation from glycogen. The low glycolytic values observed in the experiments in which no coenzymes were added are probably due to the high dilution of the coenzymes. As Jowett and Quastel [1933] have observed, lysis and high dilution of the erythrocytes may cause a complete inhibition of the activity of their methylglyoxalase which may be restored by the addition of reduced glutathione.

In the main these experiments corroborate the previous series made with tissue extracts; reduced glutathione added to the macerated tissues brought about a strong activation of lactic acid production from glucose. The activating effect of added reduced glutathione was of about the same magnitude as that of added adenosinetriphosphoric acid. The largest activation was observed when both reduced glutathione and adenosinetriphosphoric acid were added to the tissue suspensions. The activating effect of reduced glutathione on the lactic acid production from glucose was clearly evident in all the tissues examined. In the experiments made with liver tissue the effect of adenosinetriphosphoric acid was not examined. Reduced glutathione had no activating effect at all on lactic acid production from glycogen. On the contrary a small inhibiting effect is seen in the experiments made with skeletal muscle.

Table II.

			mg. lactic acid formed by 100 g. tissue per hour			
Exp. No.	Tissue	Substrate	Without activator	With adenosine-triphosphoric acid	With adenosine-triphosphoric acid and glutathione	With glutathione without adenosine-triphosphoric acid
	Brain hemispheres:					
1	0·358 g. to each bottle, total vol. 10 ml.	Glucose	55	179	500	188
2	0·358 g. to each bottle, total vol. 10 ml.	„	81	304	537	267
3	0·358 g. to each bottle, total vol. 10 ml.	„	67	288	566	300
4	0·441 g. to each bottle, total vol. 10 ml.	„	104	200	340	—
		Glycogen	14·1	8	10	—
5	0·400 g. to each bottle, total vol. 10 ml.	Glucose	60	189	370	—
		Glycogen	10	11	8	—
	Skeletal muscle:					
6	0·4 g. to each bottle, total vol. 10 ml.	Glucose	—	83	123	—
		Glycogen	—	898	778	—
7	0·4 g. to each bottle, cont. total vol. 10 ml.	Glucose	—	67	112	—
		Glycogen	—	1120	908	—
8	0·4 g. tissue, total vol. 10 ml.	„	—	1404	1216	—
9	0·4 g. tissue, total vol. 10 ml.	„	—	1160	1022	—
	Liver tissue:					
10	0·4 g. total vol. 10 ml.	Glucose	360	—	—	508
11	Do.	„	211	—	—	459
12	Do.	„	178	—	—	285

A somewhat unexpected result of these experiments is the activating effect of adenosinetriphosphoric acid on the lactic acid production from glucose by brain tissue. Cozymase and adenosinetriphosphoric acid are generally considered to belong to the esterifying enzyme system. But according to Ashford and Holmes [1929] glucolysis (from glucose) by brain tissue is independent of the presence of inorganic phosphate. According to Bumm and Fehrenbach [1931] cozymase does not accelerate brain glycolysis.

An attempt to explain these seemingly contradictory results will be made below.

Activating effect of reduced glutathione in relation to its concentration.

The quantitative relationships of the activating effect of glutathione on lactic acid formation from methylglyoxal have been investigated by Lohmann [1932]. He found, that the maximum activation is reached when the concentration of reduced glutathione is about 40 mg. per 100 ml., but even with 20 mg./100 ml. the maximum activation is approached. In the present experiments made on brain tissue about the same quantitative relation has been found for lactic acid formation from glucose. In Table III two such experiments out of five similar ones are reported. These experiments were made exactly in the same way as those described in Table II. To all the bottles adenosinetriphosphoric acid was added in the usual concentration.

Table III.

	Concentration of reduced glutathione mg./100 ml.	mg. lactic acid formed by 100 g. tissue per hour
Exp. 1. Brain tissue, rats:		
Glucose without added reduced glutathione	4·5	215
Substrate with added reduced glutathione	14·5	395
Substrate with added reduced glutathione	19·5	500
Substrate with added reduced glutathione	24·5	578
Substrate with added reduced glutathione	39·5	567
Exp. 2. Brain tissue, rats:		
Glucose without added reduced glutathione	4·0	227
Substrate with added reduced glutathione	14·0	457
Substrate with added reduced glutathione	24·0	533
Substrate with added reduced glutathione	34·0	530
Glycogen substrate, muscle tissue:		
Without added reduced glutathione	5·0	1212
With added reduced glutathione	15·0	1024
With added reduced glutathione	25·0	1075
With added reduced glutathione	45·0	971

These experiments show that the concentration of reduced glutathione necessary for full activation of the glucolysis by chopped brain tissue lies between 20 and 25 mg./100 ml.

The last experiment made on chopped skeletal muscle shows again a small but distinct inhibitory effect of reduced glutathione on lactic acid formation from glycogen.

The inhibitory effect of oxidised glutathione on glycolysis.

The indispensability of reduced glutathione for the glycolysis of glucose observed in the previous experiments offered a probable explanation of the so-called Pasteur-Meyerhof reaction.

In a recent paper Giršavicius [1933] reported that moderate access of oxygen caused rapid inactivation of glyoxalase. Bumm and Appel observed [1932] that the aerobic glycolysis of tumour tissue can be raised almost to the anaerobic level by addition of reduced glutathione. In view of the fact that reduced glutathione becomes slowly oxidised if agitated with oxygen in slightly alkaline solution it was supposed that the loss of reduced glutathione by oxidation might be responsible for the inhibitory effect of oxygen on glycolysis. In two experiments the effect of an excess of reduced glutathione in the presence of oxygen was examined. Quantities of reduced glutathione were added such that at the end of the incubation period each bottle still contained sufficient reduced glutathione to cause a full activation of glycolysis. The technique of these experiments was similar to that of the previous ones.

The flasks containing the tissue suspension, buffer solution together with glucose and the usual concentration of adenosinetriphosphoric acid, were divided into four groups. One of them to which no reduced glutathione was added was incubated anaerobically, the second one similarly prepared was incubated in oxygen. The third group to which an excess of reduced glutathione had been added was incubated anaerobically and the fourth group, similar to the third, was incubated in the presence of oxygen. At the end of the incubation period quantitative estimations of reduced glutathione were made in addition to the lactic acid estimations. Of the three experiments all giving similar results one is reported here (Table IV).

Table IV.

	mg. lactic acid produced by 100 g. tissue per hour	Final conc. of reduced glutathione in mg./100 ml.
Brain tissue without added glutathione in N_2	202	7
,, without added glutathione in O_2	82	Hopkins's reaction positive
,, with added glutathione in N_2	390	67
,, with added glutathione in O_2	76	24
,, without added glutathione in H_2O_2*	82	0
,, with added glutathione in H_2O_2*	72	11

* 0·2 ml. of 1 % solution of H_2O_2 was added to each 2 ml. of brain suspension (containing 0·4 g. of tissue) before it was introduced into the flasks. These flasks were filled with N_2.

The experiments described in Table IV show that molecular oxygen inhibits glycolysis even when an excess of reduced glutathione is present. The addition of small amounts of H_2O_2 causes the same inhibiting effect as molecular oxygen. It is thus evident that the inhibiting effect of molecular oxygen on glycolysis cannot be ascribed to the absence of reduced glutathione.

The next experiments show clearly that the inhibiting factor in the Pasteur-Meyerhof reaction is oxidised glutathione. The technical arrangements of these experiments were like those of the previous ones. To some of the bottles various quantities of oxidised glutathione were added at the beginning of the experiments. All bottles but one were incubated anaerobically. At the end of the experiments the nitroprusside reaction showed that during the incubation period a part of the oxidised glutathione had been reduced by the brain tissue. One of three uniform experiments is reported in Table V. Glucose was the substrate.

Table V.

	mg. lactic acid produced by 100 g. tissue per hour	Nitroprusside test at the end of the experiment
Brain tissue without added glutathione in N_2	282	+
,, with 10 mg./100 ml. oxidised glutathione in N_2	171	+ + +
,, with 20 mg./100 ml. oxidised glutathione in N_2	168	+ + +
,, with 40 mg./100 ml. oxidised glutathione in N_2	146	+ + +
,, without added glutathione in O_2	162	±

In these experiments 10 mg./100 ml. of oxidised glutathione cause about the same inhibition of glycolysis as the presence of oxygen. The inhibiting effect of oxidised glutathione on glycolysis is still more marked in the experiment with muscle tissue and with glycogen as substrate.

The next experiment on finely cut muscle tissue and with glycogen was made exactly like the previous ones. One of the three experiments which all gave the same result is given in Table VI.

Table VI.

	mg. lactic acid produced from glycogen by 100 g. tissue per hour	Nitroprusside test at the end of the experiment
Muscle tissue without added glutathione in N_2	1824	+
,, with 10 mg./100 ml. oxidised glutathione in N_2	810	+ + +
,, with 20 mg./100 ml. oxidised glutathione in N_2	132	+ + +
,, with 40 mg./100 ml. oxidised glutathione in N_2	74	+ + +
,, without added glutathione in O_2	1762	+

These experiments on muscle tissue show a powerful inhibitory effect of oxidised glutathione. In the presence of 40 mg. of oxidised glutathione inhibition of glycolysis is nearly complete. It is significant that in these experiments the inhibition of glycolysis caused by oxygen is very small in comparison with that caused by oxidised glutathione. It is to be observed too that in all the bottles at the end of the incubation a strong nitroprusside reaction was observed which indicated that a part of the added oxidised glutathione was reduced during the incubation period. The smallness of the inhibitory effect of oxygen in these experiments may thus be due to the reducing power of the tissue which is probably able to reduce quickly the oxidised glutathione slowly formed by the action of molecular oxygen.

With brain tissue and with glucose as substrate equal inhibition is caused by oxygen and by oxidised glutathione.

According to these experiments the inhibiting effect of molecular oxygen on glycolysis can be explained by the formation of oxidised glutathione which is directly capable of inhibiting glycolysis.

The rôle of the inorganic phosphorus in glycolysis of glucose.

According to Ashford and Holmes [1929] brain glycolysis from glucose proceeds in the absence of inorganic phosphates. It was therefore disconcerting to find that adenosinetriphosphoric acid activates the glucose breakdown by brain tissue. It was therefore decided to repeat the experiments of Ashford and Holmes and to investigate the effect of adenosinetriphosphoric acid and of reduced glutathione on tissue emulsions freed from inorganic phosphates.

At first the effect of the borate buffer alone on glycolysis was investigated. A borate buffer of p_H 8·1 was prepared according to Sørensen.

The bottles were set up as follows:

Phosphate or borate buffer p_H 8·1	5·0 ml.
2 % glucose solution	1·5 ml.
Brain emulsion in 0·9 % NaCl solution ...	2·0 ml. containing 0·3 g. of brain tissue
1·2 % $CaCl_2$ solution (or water)	1·0 ml.
Adenosinetriphosphoric acid solution (or water)	0·5 ml.

One of the two parallel experiments is reported in Table VII.

Table VII.

	mg. lactic acid produced by 100 g. tissue in 1 hour
Phosphate buffer	320
Phosphate buffer + adenosinetriphosphoric acid	540
Borate buffer	18
Borate buffer + adenosinetriphosphoric acid	29
Borate buffer + adenosinetriphosphoric acid + $CaCl_2$	130

These experiments show that borate itself strongly inhibits glycolysis. Adenosinetriphosphoric acid does not counteract this inhibition but strangely enough $CaCl_2$ does. To investigate this effect of $CaCl_2$ more experiments were set up with different amounts of $CaCl_2$.

The bottles were set up as follows:

Borate buffer p_H 8·2	5 ml.
3 % glucose solution	1 ml.
1·2 % $CaCl_2$ solution (or water)	3 ml.
Adenosinetriphosphoric acid (or water)	0·5 ml.
Reduced glutathione solution (or water)	0·5 ml.

The experiments were made anaerobically in every respect like the previous experiments. One of the two similar experiments is reported in Table VIII.

Table VIII.

	mg. lactic acid produced by 100 g. tissue per hour
Borate buffer without $CaCl_2$	28
„ with 0·5 ml. $CaCl_2$	51
„ with 1·0 ml. $CaCl_2$	111
„ with 2·0 ml. $CaCl_2$	174
„ with 3·0 ml. $CaCl_2$	175
„ with 2·0 ml. adenosinetriphosphoric acid	196
„ with 2·0 ml. adenosinetriphosphoric acid + glutathione	297

These experiments show that $CaCl_2$ counteracts the inhibiting effect of borate and that in the presence of borate and of $CaCl_2$ and thus in the absence of inorganic phosphate the activating effect of reduced glutathione is still very evident. These experiments also show that under the experimental conditions described so many factors introduce complications that it is difficult to estimate the rôle of the inorganic phosphorus in brain glycolysis. It seems, however, in agreement with Ashford and Holmes [1929] that the presence of inorganic phosphorus is not essential in this process.

A further fact of interest is that while the activating effect of adenosinetriphosphoric acid disappears under these conditions, the activating effect of glutathione is not altered. This phenomenon may suggest two mechanisms of glucose breakdown in the brain, one of which does not need the presence of inorganic phosphate and is activated by reduced glutathione and a second one which only proceeds in the presence of inorganic phosphate and of adenosinetriphosphoric acid. This may explain also why adenosinetriphosphoric acid activates brain glycolysis independently of glutathione.

A comparison of the glyoxalase activities of brain and muscle tissue.

In the previous pages it was suggested as possible that methylglyoxal may be an intermediary product of the breakdown of glucose but not of glycogen. It is to be expected accordingly that those tissues which produce lactic acid from glucose must be able to transform a correspondingly large quantity of methylglyoxal into lactic acid. On the other hand if methylglyoxal is not an intermediary product in lactic acid formation from glycogen those tissues in which lactic acid formation from glycogen is predominant should be unable to form a correspondingly large quantity of lactic acid from methylglyoxal.

The rats selected for these experiments were kept under strictly identical conditions at least for three generations. The technique of these experiments was described in the general part. The contents of the bottles were as follows:

Phosphate buffer solution p_H 6·95	5 ml.
Methylglyoxal 0·7 %	3 ml.
Tissue suspension containing 0·4 g. tissue	2 ml.

In the experiment with muscle tissue 0·4 g. of finely cut muscle was weighed into every bottle and 1·6 ml. of water were added. The methylglyoxal solution was accurately neutralised before addition. Four bottles were set up for each experiment as described above. Table IX shows the result.

Table IX shows that muscle tissue which is able to form on the average 1176 mg. lactic acid per 100 g. tissue per hour from glycogen and 101 mg. from

Table IX.

	mg. lactic acid produced by 100 g. tissue in 1 hour		
	From methylglyoxal	From glucose	From glycogen
Muscle tissue	242	—	1160
	278	—	1022
	236	108	—
	197	83	—
	221	123	—
	215	79	1212
	309	—	1024
	302	112	1462
Average	**251**	**101**	**1176**
Brain tissue	1487	326	28
	1328	308	19
	1384	320	—
	1222	—	14
	1247	340	18
	1055	296	—
Average	**1287**	**318**	**19**

glucose, forms only 251 mg. lactic acid from methylglyoxal. As a general rule it is to be expected that the conversion of an intermediary product should proceed at least with the same velocity as that of the initial product. Actually the transformation of glycogen into lactic acid in the muscle proceeds 4–5 times as rapidly as that of methylglyoxal. Thus methylglyoxal cannot possibly be an intermediary product in the breakdown of glycogen.

Brain tissue, on the other hand, which produces lactic acid mainly from glucose, is able to convert a large quantity of methylglyoxal. For 318 mg. of lactic acid formed from glucose, the brain tissue is able to form 1287 mg. of lactic acid from methylglyoxal.

Discussion.

The experiments contained in the first part of this paper demonstrate that the enzyme system which produces lactic acid from glycogen is unable to form lactic acid from glucose. While reduced glutathione is essential in the process of lactic acid production from glucose, glycogen can be split into lactic acid in its absence. Reduced glutathione added to tissue suspensions greatly accelerated the lactic acid formation from glucose but inhibited it to a small extent when glycogen was the substrate.

By the fact that reduced glutathione is a common coenzyme of the methylglyoxalase and of the glycolytic enzyme system, the probability of methylglyoxal being an intermediary product in the formation of lactic acid from glucose is strengthened. The concentrations of reduced glutathione necessary for the full activation of the glycolysis (of glucose) and of methylglyoxalase are similar.

On the other hand it is hardly possible to consider methylglyoxal as an intermediary in the process of lactic acid formation from glycogen, as this process proceeds also in the absence of glutathione. The experiments of Embden *et al.* [1933] of Meyerhof and Kiessling [1933] and of Meyerhof and McEachern [1933] excluded the intermediary formation of methylglyoxal from the lactic acid production from glycogen and hexosediphosphate by muscle. It is also known that muscle tissue splits mainly glycogen, glucose only to a small extent.

On the basis of these facts the hypothesis is ventured that glucose breakdown in the cells proceeds with the intermediary formation of methylglyoxal, in accordance with the theory of Neuberg, while the breakdown of glycogen proceeds as postulated by Embden *et al.* and Meyerhof *et al.* through pyruvic acid. A recent paper of Barrenscheen and Bereschkovsky [1933], according to which tissues other than muscle are unable to produce pyruvic acid from phosphoglyceric acid, also points in this direction.

The inhibiting action of oxidised glutathione is of peculiar interest as offering an explanation of the Pasteur-Meyerhof reaction. It was found formerly by Lipmann [1933] that oxidising agents such as iodine and quinone inhibited the glycolysis of muscle extracts. The glycolysis of these extracts was also reversibly inhibited by O_2 in the presence of a redox indicator, 2:6-dichlorophenolindophenol. Lipmann therefore concluded, that the Pasteur reaction is an oxidative inhibition of the glycolysis, as the glycolytic ferment of the cells becomes inactivated by oxygen transmitted by the autoxidisable respiratory enzyme.

According to the present experiments oxidised glutathione may be considered as the transmitter of oxygen to the glycolytic enzyme. Reduced glutathione can be easily oxidised by molecular oxygen in a weakly alkaline medium. In its turn, oxidised glutathione proved to be a powerful inhibitor of glycolysis.

Since in muscle glycolysis from glycogen no reduced glutathione is necessary and since the inhibitory effect of oxidised glutathione persists also in the presence of reduced glutathione it is evident that reduced glutathione in this case may act only as an intermediary by transmitting oxygen to the glycolytic enzyme.

On the other hand in the glycolysis of glucose the oxidation of glutathione has a double significance. First, by oxidation of reduced glutathione the glycolytic enzyme system is deprived of one of its coenzymes, and secondly, the presence of oxidised glutathione inactivates the glycolytic enzyme. It is to be noted that in the present experiments the quantitative aspect of the inhibition of these two types of glycolysis by oxidised glutathione has not yet been sufficiently considered. It seems, however, that a more complete inhibition can be obtained by oxidised glutathione in the breakdown of glycogen than in that of glucose.

No explanation can be offered for the observed actions of borate and of calcium on glycolysis.

The experiments on the glyoxalase activity of muscle and brain tissue provide further evidence in favour of the theory that methylglyoxal cannot be considered as intermediary in glycogen breakdown but is a possible intermediary in glucose breakdown.

SUMMARY.

1. Tissue extract from which reduced glutathione is removed cannot convert glucose into lactic acid. The addition of reduced glutathione restores this ability to the extracts.

2. Reduced glutathione added to tissue suspensions accelerates the rate of lactic acid formation from glucose but inhibits to a small extent that from glycogen.

3. Oxidised glutathione even in low concentration inhibits lactic acid formation from glucose as well as from glycogen.

4. Borate inhibits brain glycolysis, $CaCl_2$ restores it to a small extent.

5. It is concluded that reduced glutathione is a coenzyme of the glycolytic enzyme system and that lactic acid production from glucose may proceed through methylglyoxal as an intermediary, while this possibility is excluded in the case of glycogen.

6. An explanation is offered for the Pasteur-Meyerhof reaction.

REFERENCES.

Ashford (1933). *Biochem. J.* **27**, 923.
—— and Holmes (1929). *Biochem. J.* **23**, 748.
Barrenscheen and Bereschkovsky (1933). *Biochem. Z.* **265**, 159.
—— and Dreguss (1931). *Biochem. Z.* **233**, 305.
—— and Filz (1932). *Biochem. Z.* **250**, 281.
Bumm and Appel (1932). *Z. physiol. Chem.* **210**, 79.
—— and Fehrenbach (1930). *Z. physiol. Chem.* **193**, 238.
—— —— (1931). *Z. physiol. Chem.* **195**, 101.
Case and Cook (1931). *Biochem. J.* **25**, 1319.
Embden, Deutike and Krafft (1933). *Klin. Woch.* 213.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
—— and Graeser (1933). *J. Biol. Chem.* **100**, 291.
Giršavicius (1933). *Biochem. J.* **27**, 537.
Haarmann (1932). *Biochem. Z.* **255**, 203.
Jowett and Quastel (1933). *Biochem. J.* **27**, 486.
Kuehnau (1931). *Biochem. Z.* **230**, 353.
Lipmann (1933). *Biochem. Z.* **265**, 133.
Lohmann (1932). *Biochem. Z.* **254**, 332.
Meyerhof and Kiessling (1933). *Biochem. Z.* **264**, 60.
—— and McEachern (1933). *Biochem. Z.* **260**, 417.
Neuberg and Hofmann (1930). *Biochem. Z.* **224**, 491.
Pirie (1930). *Biochem. J.* **24**, 51.
Watson and Mitolo (1934). *Biochem. J.* **28**, 811.

XCVI. RAPID TITRIMETRIC ESTIMATION OF ARSENIC IN BIOLOGICAL MATERIAL.

BY RUTH ALLCROFT AND HENRY HAMILTON GREEN.

From the Veterinary Laboratory, Ministry of Agriculture, Weybridge.

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IN the course of work upon the fate of arsenic in avian metabolism the need arose for a rapid method of estimating quantities of arsenic from 1 mg. down to 0·005 mg. As_2O_3 per 10 g. sample of biological material, with a probable error not exceeding ± 5 % for quantities around 0·005 mg. In a balance experiment on a laboratory animal in which it is desired to account quantitatively for administered arsenic as eliminated in faeces and urine or retained in various tissues, such a degree of accuracy meets most demands for physiological information; but wider errors are objectionable and in our opinion most of the older statements regarding prolonged retention of considerable quantities of arsenic in the body arose from faulty recovery of excreted arsenic and the unjustifiable assumption that the unrecovered arsenic was still in the tissues.

The range of accuracy arrived at is also satisfactory for public health administration concerning arsenic in foods where 1·4 parts per million is the permissible figure suggested by the Royal Commission of 1903.

A survey of existing procedures revealed laborious methods sufficiently accurate and rapid methods open to higher error, but despite the voluminous literature on arsenic determination it seemed probable that a more serviceable routine could be devised. The present communication claims to supply one which is effective with a few γ of material but has an "average working time" of only 20 mins. per determination in a routine series of half a dozen analyses. Since it involves a wet combustion, a separation as arsine and a final microtitration, a single determination cannot very well be completed in less than 90 mins., but since a rack of six blood analyses can be finished in 2 hours the procedure becomes as simple as the Kjeldahl process for total nitrogen. The economy of time is effected by combining a very rapid perchloric-nitric acid wet combustion with a rapid subsequent separation of arsine in a fast stream of hydrogen, absorption in dilute silver nitrate and direct micro-titration of the liberated arsenious acid with iodine in presence of sufficient potassium iodide to retain excess silver in clear solution and so render possible a delicate starch end-point. The iodine is arranged to express results directly on the burette and obviate arithmetic (1 ml. $N/495 = 0{\cdot}1$ mg. As_2O_3 or 1 ml. $N/375 = 0{\cdot}1$ mg. As).

Discussion of existing methods.

Since arsenic determination for biological purposes usually involves destruction of large amounts of organic matter and subsequent estimation of real quantities below 0·1 mg. ordinary gravimetric and volumetric methods are excluded and colorimetric or nephelometric methods and micro-titration or mirror-matching technique come into consideration. For literature on such methods the reader is referred to Heller [1934] who, in collecting references on micro-methods since 1928, cites 54 publications dealing with arsenic; to Kleinmann and Pangritz [1927] who give numerous references to previous technique; to

Minot [1926] who does the same and to Cox [1925] who concerns himself chiefly with English public analyst requirements and with checking the methods of Bang and Ramberg.

The precise technique of combustion is important since small losses are liable to occur both in wet and dry methods, and it is usual to effect a preliminary separation of arsenic from the combustion residue either as arsine or as arsenic trichloride before proceeding to actual estimation. Where the quantity of arsenic can be arranged to be relatively large (say 5 mg.) as in estimations on organic arsenicals the very simple method of Ewins [1916] is serviceable. Here the organic matter is destroyed with sulphuric acid as in an ordinary Kjeldahl combustion, with concomitant reduction of arsenic to arsenious acid, the excess sulphuric acid neutralised with soda lye and finally with sodium bicarbonate and the arsenic titrated directly with iodine. But where the amount of arsenic is small loss of traces of it by volatilisation and the possible presence in the combustion residue of substances which liberate iodine invalidate the procedure. Nitric-sulphuric acid combustion, in which the bulk of the organic matter is destroyed at lower temperature, is the generally recognised procedure and the technique of Ramberg [see Cox, 1925] is preferable to that described in the Report of the Conjoint Committee on the detection and approximate estimation of minute quantities of arsenic [1902]. The American Association of Official Agricultural Chemists [1930] also adopts combustion with nitric and sulphuric acids. Scott [1925] gives the unsatisfactory hydrochloric acid and potassium chlorate method. Hitherto no one seems to have recorded trials of perchloric acid with nitric or sulphuric acid. Amongst the little used dry methods, combustion with magnesium nitrate, as described below, would seem to be satisfactory.

In most cases where the nitric-sulphuric acid wet combustion is used it is now customary to remove residual traces of nitric acid by dilution, addition of oxalate and recombustion—a procedure which adds to the working time but is necessary when ensuing separation of arsenic is to be as trichloride.

Minot [1926] then proceeds to final determination without separation of the arsenic, reducing to trivalent form by saturating the diluted combustion residue with SO_2, expelling this by evaporation and finally titrating direct with *N*/500 iodine. Blanks, however, amount to 0·01 mg. As_2O_3 and vary ± 20 % (0·08 ml.–0·12 ml.), and the time saved in separating as AsH_3 or $AsCl_3$ is lost by reduction with SO_2 and final evaporation.

Cox [1925], the English Sub-committee on the determination of arsenic in food colouring materials [1930], von Fellenberg [1930] and most recent workers agree that arsenic must be separated from the combustion residue before final determination. Cox considers that separation by distillation of the trichloride [Bang, 1925] followed by micro-titration with standard potassium bromate as used by Ramberg is satisfactory if the amount of arsenic to be determined is not less than 0·01 mg.; below this he recommends the Marsh-Berzelius method following the Ramberg technique for destruction of organic matter. Nevertheless the personal trials of Cox with the bromate titration, using pure carbohydrate with known quantities of arsenic, gave recoveries of 0·06 mg. in place of 0·05 mg. (20 % too high), 0·03 mg. in place of 0·02 mg. (50 % too high) and 0·02 mg. in place of 0·01 mg. (100 % too high). The results from 0·05 mg. downwards are thus much too high and this is a more serious fault than being too low; in the latter case empirical correction can be justified if duplicates are good and small losses consistent.

Kleinmann and Pangritz [1927] regard colorimetric methods of the Gutzeit type as inherently unreliable and consider that amongst the titrimetric methods the technique of Billiter [1923], who dissolves the Marsh mirror in iodine and back-titrates with thiosulphate, is the most accurate. They themselves describe a nephelometric method sensitive to 0·5γ H_3AsO_4 in pure solutions and accurate to 5 % even with quantities below 0·01 mg. The principle of the method is precipitation with cocaine molybdate and nephelometric determination of turbidity; previous combustion being carried out by the nitric-sulphuric method of Ramberg and essential separation of arsenic being effected by the trichloride distillation of Bang [1925]. Unfortunately their recovery figures refer mainly to direct nephelometric determination on pure solutions and insufficient data are supplied on the operation of the whole process with tissues to which known amounts of arsenic have been added in order to test the summation of errors involved in combustion, distillation, precipitation and nephelometric matching.

Amongst newer colorimetric methods may be mentioned those of Maechling and Flinn [1930] and of Youngburg and Farber [1932]. The former use nitric-sulphuric combustion followed by distillation of arsenic trichloride, convert into quinquivalent form by evaporating with nitric acid and bromine, heat with reduced molybdate and compare in a colorimeter with an arsenate standard. The latter precipitate as arsenic sulphide, oxidise this and determine colorimetrically with molybdate and stannous chloride. But no recovery tables for arsenic added to tissues are given in either of these papers.

Von Fellenberg [1930] also precipitates as sulphide (with H_2S after distilling as $AsCl_3$ from the combustion residue) but titrates the isolated sulphide with permanganate in alkaline solution. Good results are shown even with a few γ but unfortunately in recovering added arsenic (limited data) the smallest magnitudes are returned too high and the higher magnitudes too low so that there is the suggestion of arbitrary compensatory errors just at the point (0·01 mg.) where recoveries by other methods begin to be unduly low.

On the question of separation of arsenic as AsH_3 by zinc and sulphuric acid most authors agree that recovery is good with pure solutions of arsenic provided that a properly activated pure zinc is used, but some consider that it may be rendered imperfect by substances in the combustion residue and therefore recommend a preliminary separation as trichloride even when subsequent arsine separation follows incidentally on the use of Gutzeit papers. This double separation occurs in the method described in the First Report of the Sub-committee on determination of arsenic in food colouring materials [1930], although Cox [1925] recommends the Marsh-Berzelius procedure direct on the Ramberg combustion residue.

Despite the variety of terminal procedures at disposal the most popular finishing estimation is still the colorimetric comparison of Gutzeit papers. But Neller [1929] in his study of the accuracy of the Gutzeit procedure considers the inherent error too high and the English Sub-committee, while regarding the stain produced by 0·005 mg. As_2O_3 as "a very suitable intensity to evaluate", concedes that "a stain actually due to 0·0045 mg. may be estimated at 0·004 or 0·005 according to individual judgment," and that this 20 % difference may appear equally in stating results of 1 part or 20 parts per million, irrespective of the real magnitude of the quantity in the analysed aliquot. Although the procedure is sound enough for most ordinary needs it might well be considered that the tedious combustion, dilution and reconcentration, distillation as trichloride and second separation as arsine, are labour lost if they end with an inherent error of ± 10 % irrespective of whether 0·20 mg. or 0·01 mg. As_2O_3 per 10 g. sample is dealt with. The significance of double separation of arsenic is worthy of further investigation but the usefulness of abbreviating combustion needs no defence, and the obviation of individual judgment is still more desirable. Since a starch-iodine end-point is as delicate as an arsenic-mercury stain we believe that microtechnique is capable of carrying titration as far as Gutzeit papers. If recovery of arsenic is then incomplete but duplicates agree well, and an empirical upward correction is indicated, varying with the real magnitude of the arsenic at disposal, the correction is at least deliberately made and not unconsciously incorporated.

The proposed method.

The combustion procedure is essentially that described by Bolliger [1932] for general use in destroying organic matter by means of perchloric and nitric acids. The use of sulphuric acid in place of nitric (1 ml. perchloric acid, 1 ml. sulphuric acid per 0·2 g. of dry matter) associated with higher combustion temperature, as used by McCance (personal communication) for subsequent colorimetric determination of total phosphorus in foods, is equally serviceable and even speedier when the quantity of organic matter is sufficiently small for the analyst to view possible conflagration in the combustion vessel with equanimity, but the lower boiling-point of the perchloric-nitric mixture is an advantage when large combustion volumes have to be considered in relation to traces of arsenic liable to loss by volatilisation. The need for secondary dilution, addition of oxalate and recombustion to get rid of residual nitric acid, disappears since separation as $AsCl_3$ does not follow. The technique of the arsenic titration is a refinement of that used by Green [1918] in which the familiar reaction

$$2AsH_3 + 12AgNO_3 + 3H_2O = 12Ag + 12HNO_3 + As_2O_3$$

is used; precipitation of silver iodide is held up by addition of excess potassium iodide and the arsenic is directly titrated with iodine. It is sufficient to add the observations (*a*) that perchloric acid does not interfere with the liberation of arsine by zinc and sulphuric acid, (*b*) that absorption of traces of arsine from large volumes of hydrogen by silver nitrate as dilute as $N/100$ is complete even when the gas is blown through a few cm. depth of liquid at the rate of several litres per minute, and (*c*) that the starch end-point is still sensitive to less than 1γ when soluble iodides are present in large excess so that micro-titration can take the place of the conventional Gutzeit papers and obviate the personal equation altogether. In general 10 g. of wet tissue form a suitable aliquot and since 1 ml. $N/495$ iodine $= 0·1$ mg. As_2O_3 the microburette reading gives mg. per 100 g. without further calculation.

Reagents. A.R. quality and As-free materials should be used throughout.

Perchloric acid 60 %.
Nitric acid, conc.
Stannous chloride solution. Dissolve 40 g. $SnCl_2, 2H_2O$ in HCl and make up to 100 ml. with the same strength acid [Assoc. Off. Agric. Chem. 1930].
Copper sulphate 5 %.
Lead acetate 1 %.
Silver nitrate, approximately $N/50$.
Starch solution 1 % (fresh).
$N/495$ iodine, freshly diluted from stock $N/10$ (2 ml. $N/10$ made up to 99 ml.).
Sodium bicarbonate.
Potassium iodide.
Zinc shot (tested for interfering impurities).
Sulphuric acid.
Standard arsenic solution. 1 ml. $= 0·1$ mg. As_2O_3 for intermittent checking of process.

Moir and Jamison [1925] have described an electrolytic generator for use with the titration method of Green [1918] but zinc and acid generators are more usual and more conveniently prepared in large numbers.

Perchloric acid frequently contains traces of arsenic (up to 0·75 mg. per litre) and although it is possible to obtain it As-free (B.D.H. supply usually is so) a sample should be procured before purchasing in bulk and a test made to ensure that it will not introduce too high a blank. As-free zinc shot is easy to obtain but should be tested in the actual process to ensure that it permits good recovery of arsenic from the standard solution. Samples have been encountered which contain no arsenic but which give low results, apparently by retaining traces of arsenic in the generator. In making this test a suitable strength of sulphuric acid to use in the generator is 1: 5, with catalysts as usual. It should be needless to point out that glassware often contains arsenic and that for combustion flasks exposed to high temperature and strong acid mere preliminary "pickling" is not enough. We have found monax glass quite suitable.

First stage. 10 g. of finely divided wet tissue or 2–5 g. dry matter are introduced into a 300 ml. Kjeldahl flask and covered with 20 ml. of perchloric acid. The quantity of perchloric acid (which should be noted if not quite As-free) naturally depends upon the material but to avoid the risk of explosion it is essential to err on the side of liberal excess. In the case of dilute liquids such as urine preliminary concentration by boiling precedes the addition of perchloric acid. If dry matter is used care should be taken to ensure thorough wetting with the acid before heating, and for adhesive powders such as flour it is best to wet to a paste with water first. Speculative adjustment of sample aliquots on the basis of anticipated real arsenic values naturally facilitates any final corrections which may have to be made. In the case of material rich in arsenic 1 ml. or 1 g., or less, may be taken and the scale of operation reduced to a test-tube 1 × 7 in. which, if desired, can also serve as subsequent Marsh generator for a reduced scale absorption apparatus.

Two glass beads are added to facilitate even boiling and the mixture is brought to ebullition and appearance of perchloric fumes over a bare flame. The flask is then transferred to a cork ring in the combustion cupboard and concentrated nitric acid added from a dropping pipette. Vigorous reaction sets in almost at once but is easily controlled by adjusting the rate of addition of nitric acid. When no further reaction occurs the yellow-brown mixture is heated on a Kjeldahl or other digestion rack until white fumes of perchloric acid appear. Continuous observation is necessary to control recrudescence of reaction. When the volume is reduced to about half oily drops appear on the sides of the flask and a vigorous reaction usually occurs. If so it is necessary to turn out the flame. After the reaction subsides the digest either turns practically colourless or else dark brown. If the latter then more nitric acid must be added, in which case it is advisable to remove the flask to its cork ring and allow to cool somewhat before adding the requisite further nitric acid and restoring to the digestion stand for completion of combustion. Usually 2 or 3 ml. are sufficient as indicated by the dark brown colour changing to a yellow-brown. Complete combustion is indicated by a clear, pale, yellowish green residue associated with white perchloric fumes. The residue is usually colourless on cooling, but if not can generally be rendered so by final addition of a few drops of perhydrol. Tissues such as muscle, blood and urine give a colourless cold digest; a faeces digest may be slightly yellow.

Although the digestion process requires constant attention it is not at all laborious. The time taken for the whole combustion varies from about 10 mins. for 10 ml. of blood or urine, to about 30 mins. for 10 g. of muscle or liver tissue and over an hour for 2 g. of dried faeces, but since 6 to 12 combustions can be supervised simultaneously the actual duration of a single digestion is unimportant.

Second stage. The digest is diluted and washed into a generating flask to a total volume of about 100 ml.; 10 drops of stannous chloride solution and 2 drops of copper sulphate are added to speed up evolution of hydrogen. About 15 ml. of concentrated sulphuric acid are added and the contents heated over a flame for a few seconds if the mixture is not warm enough to ensure immediate subsequent vigorous evolution of hydrogen; about 25 g. zinc shot are then quickly added and the flask immediately connected up to the previously prepared absorption tubes (A, B, C and D). Of these, the first tube A contains 1 % lead acetate to remove any traces of H_2S formed by reduction of the hot dilute sulphuric acid. The remaining three tubes B, C, D contain $N/50$ or $N/100$ $AgNO_3$, not stronger. Two are sufficient (*i.e.* B and C) in ordinary practice so that the last tube D becomes a guard tube in case unsuspectedly large amounts of arsenic are found (over 1 mg.). For quantities of 0·2 mg. over two-thirds is caught in the first tube; for 0·1 mg. or less the last tube D is unnecessary and C serves as guard. Three or four ml. of $N/50$ $AgNO_3$ in the first two tubes cover any range up to 1 mg. As_2O_3. For quantities below 0·01 mg. $N/100$ $AgNO_3$ (approximately) is preferable.

Evolution of hydrogen from the hot acid is very rapid and the arsenic is swept over completely in 20 mins.—the faster the better so long as the liquid is not blown out of the tubes. Too slow evolution of hydrogen seems to lead to bad recoveries of arsenic.

Tubes B and C are then detached and the contents of the latter added to the former if any trace of silver deposition is noted in it. It is rarely necessary to add the contents of the guard tube. About 0·1 g. of sodium bicarbonate and about 2 g. of potassium iodide crystals, or sufficient to redissolve the silver iodide first precipitated, are added. After final addition of about 0·5 ml. of fresh 1 % starch solution $N/495$ iodine is run in to violet end-point. On a titration volume of about 5 ml. one drop from an ordinary 1 ml. micro-burette with waxed tip, corresponding to 0·01 ml. = 0·001 mg. or 1γ As_2O_3, gives a sharp end-point but this is not the limit of sensitiveness since change of violet tint can be detected with less. By reducing the titration volume to 1 ml. and using an appropriate adaptation of the Rehberg type of burette an end-point of $0{\cdot}1\gamma$ could be reached. This would come into consideration if it were desired to reduce the whole scale of technique, combust small aliquots in test-tubes and absorb the AsH_3 in, say, 1 ml. $N/100$ $AgNO_3$; but such refinement is rarely required for physiological work.

With the 1 ml. burette used in this work for quantities up to 0·1 mg. As_2O_3 each division corresponded to 1/100 ml. = 1γ As_2O_3 in the sample, and the starch end-point was taken to one division. For larger quantities a 5 ml. or 10 ml. burette was used.

Illustrative results. The method has been used extensively in physiological work over the past year with sufficiently good results but the following data may be taken to illustrate the results in routine practice, working at a speed which allowed 24 determinations to be completed in an uninterrupted 8-hour day.

Table I shows the recoveries of arsenic added to the generator flask as standard solution, *i.e.* omitting the combustion process and considering only the perfection of separation as arsine, absorption in silver nitrate and subsequent titration with iodine.

Table I.

As_2O_3 added (mg.) ...		1·0	0·5	0·2	0·1	0·05	0·02	0·01	0·005
ml. *N*/495 iodine solution required	(1)	9·67	4·87	2·03	0·985	0·505	0·205	0·110	0·055
	(2)	9·82	4·87	1·92	1·015	0·495	0·195	0·100	0·060
	(3)	9·80	4·80	1·92	0·985	0·500	0·205	0·105	0·055
Blank, ml. iodine		0·02	0·02	0·02	0·015	0·015	0·015	0·015	0·015
Average, *minus* blank		9·74	4·83	1·94	0·980	0·485	0·187	0·090	0·042
mg. As_2O_3 recovered		0·974	0·483	0·194	0·098	0·0485	0·0187	0·0090	0·0042
% of theoretical value		97	97	97	98	97	94	90	84

A few determinations were also carried out in which the two drops of copper sulphate were omitted and only the stannous chloride activator used, to ascertain whether the trace of copper helped or hindered recovery. No noteworthy difference was recorded so that the function of the copper is apparently confined to speeding up the generation of hydrogen. Another series was executed to ascertain the best concentration of silver nitrate to use. Approximately *N*/50 was found to be optimum for quantities between 0·01 mg. and 1 mg. and *N*/100 for quantities below 0·01 mg. *N*/10 was definitely too strong in the sense that recoveries were about 10 % lower than with *N*/50 but the reason for this was not specially investigated.

From Table I it will be noticed that the recoveries are very good down to 0·05 mg., much better than could be counted upon in Gutzeit colour matching, and that triplicates are satisfactory.

There is, however, a definite small loss which diminishes as the real quantity of arsenic diminishes but which is exaggerated when results are expressed on a percentage basis. Triplicates, however, agree within the limitations of the endpoint of the burette and the titration volume, and the blank is open to the same titration error, so that nothing better could be expected. This point is brought out again in Table II which represents a series of recoveries on the whole process adding known quantities of arsenic to 10 g. of liver tissue in the combustion flask, and comparing with recoveries on pure solutions.

Table II.

	Recovered			
	From pure solutions		After digestion + 10 g. liver	
As_2O_3 added in mg.	mg.	%	mg.	%
1	0·965	97	—	—
	0·980			
	0·978			
0·5	0·485	97	0·480	97
	0·485		0·485	
	0·478		0·490	
0·1	0·097	98	0·095	93
	0·100		0·091	
	0·097		0·093	
0·05	0·049	97	0·048	94
	0·048		0·045	
	0·0485		0·048	
0·01	0·0095	90	0·008	82
	0·0085		0·0085	
	0·0090		0·008	
0·005	0·0040	84	0·003	64
	0·0045		0·0035	
	0·0040		0·003	

It will be noted that down to 0·05 mg. As_2O_3 the perchloric-nitric combustion process has not involved any significant loss. At 0·05 mg. the difference between 97 % and 94 % recovery is negligible in view of the fact that 0·048 mg. recoveries appear in both sets of triplicates. There is a real loss varying between 1γ and 2γ As_2O_3; this variation in recovery is of less consequence for quantities over 0·05 mg. but is more serious when results are expressed on a percentage basis with quantities so low as 0·005 mg. The average of 0·0042 mg. recovered from the pure solution and of 0·0032 mg. recovered from the liver tissue means a loss of 16 % in one case and 36 % in the other, but one would hesitate to attribute the difference wholly to the combustion. The agreement between triplicates, however, is within the limits of titration of the volume concerned so that in a balance experiment in which it was necessary to account on the basis of 100 % recovery, an upward correction of 0·002 mg. to the uniformly low results and admission of a variation of ± 0·0005 mg. (± 10 %) in drawing conclusions, would seem to be justifiable.

A further series of trials is offered in Table III, this time on different substances to which "1·4 parts per million", the critical limit for the public health analyst, were added.

Table III. *Recoveries in parts per million and as percentage of arsenic added to various materials. 0·014 mg.* As_2O_3 *per 10 g. or 10 ml.*

Urine		Beer		Blood		Milk		Flour	
Parts per million	%	Parts per million	%	Parts per million	%	Parts per million	%	Parts per million	%
1·30	93	1·20	86	1·10	79	1·30	93	1·20	86
1·25	89	1·15	82	1·20	86	1·30	93	1·15	82
1·15	82	1·15	82	1·15	82	1·25	89	1·25	89
1·20	86	1·20	86	1·15	82	1·30	93	1·25	89

It will be observed that recoveries vary from 1·15 to 1·30 parts per million, or 82 to 93 % of the added arsenic. Most analysts would agree that this is good and report the figures direct, but since all data are low a higher degree of accuracy could be obtained by correcting upwards by 12 % and stating results within a probable error of ± 7 %.

Comparison of combustion methods. Since the method of digestion utilised here does not seem to have been discussed for arsenic before, it is considered advisable to add comparative data on different methods of combustion. Table IV gives the recoveries of known quantities of arsenic added to 10 g. minced liver tissue in each case. The methods were:

(1) Ordinary Kjeldahl combustion with 3 g. potassium sulphate and a fragment of copper as catalyst.

(2) Combustion with nitric and sulphuric acids as described in Assoc. Off. Agric. Chem. [1930, p. 307].

(3) Hydrochloric acid and potassium chlorate method as given by Scott, [1925, p. 51].

(4) Perchloric-nitric combustion as described above.

(5) Dry ashing with magnesium nitrate, a method sometimes used by Green in dealing with large quantities of rumenal contents or grass. This consists in thoroughly mixing the material with 20 % magnesium nitrate solution (about 40 ml. for 10 g. liver tissue), taking to dryness on the water-bath and ashing at moderate temperature in an electric muffle. The light white ash is then moistened with water, dissolved by warming with 1 : 5 sulphuric acid and transferred to the generator flask for completion of the determination as usual.

Table IV. *Method of digestion.*

As_2O_3 added mg.	Sulphuric acid	Nitric acid + sulphuric acid	Hydrochloric acid + potassium chlorate	Perchloric acid + nitric acid	Ashing with magnesium nitrate
0·5	77	81	49	97	90
	59	84	41	96	88
	52	77	50	98	95
Average recovery	63	81	47	97	91
0·1	61	71	53	95	95
	72	70	—	91	83
	67	85	—	93	94
Average recovery	67	75	—	93	91
0·05	70	72	48	96	92
	72	72	36	90	84
	66	74	—	96	88
Average recovery	69	73	42	94	88
0·01	90	70	40	80	85
	50	70	50	85	70
	60	80	—	80	80
Average recovery	67	73	45	82	78
0·005	Nil	50	Nil	60	50
	40	70	Nil	70	40
	20	50	Lost	60	50
Average recovery	20	57	Nil	63	47

The combustion with hydrochloric acid and potassium chlorate was so troublesome and unsatisfactory that it was not considered worth completing the series. The high temperature sulphuric acid combustion obviously leads to material percentage losses even with quantities as high as 0·5 mg. Dry combustion with magnesium nitrate appears to be as satisfactory as nitric-sulphuric digestion and may occasionally be of service with certain classes of material. In using it abundant excess of magnesium nitrate should be used and care taken to ensure thorough permeation of the sample. The nitric-sulphuric combustion might have given higher recoveries, especially on the 0·5 mg. and 0·1 mg. quantities, if the precautions emphasised by Cox in discussing Ramberg's procedure had been studiously observed, but the Assoc. Off. Agric. Chem. [1930] method was purposely carried out as it would be in ordinary laboratory practice. The run of results with the perchloric-nitric combustion is obviously best of all so that it appears to have in its favour superiority of recovery as well as greater speed of destruction of organic matter. This is attributed to the lower boiling-point of perchloric acid as compared with sulphuric at the last stage of combustion, as well as to the shorter period at high temperature.

SUMMARY.

A rapid quantitative method for determination of arsenic in biological material is described which enables six determinations to be completed in about 2 hours. A mixture of perchloric and nitric acids is used for destruction of organic matter. Arsenic is liberated as arsine in a fast stream of hydrogen, absorbed in a few ml. of $N/50$ silver nitrate and directly titrated with $N/495$ iodine (1 ml. = 0·1 mg. As_2O_3) to starch end-point in presence of sufficient excess of potassium iodide to keep silver in solution. Micro-titration is regarded as superior to matching Gutzeit papers, the inherent error of which may operate indifferently over large and small real quantities of arsenic.

Recoveries of added arsenic in quantities of 0·50 mg. to 0·005 mg. As_2O_3 compare favourably with recoveries by other methods. Duplicates are good and probably validity of results can be improved by an upward correction for consistent small losses.

REFERENCES.

Association of Official Agricultural Chemists (1930). Methods of analysis. Third ed. p. 307.

Bang (1925). *Biochem. Z.* **161**, 193.

Billiter (1923). *Helv. Chim. Acta*, **6**, 771.

Bolliger (1932). *Austral. J. Exp. Biol. Med. Sci.* **10**, 57.

Cox (1925). *Analyst*, **50**, 3.

Ewins (1916). *J. Chem. Soc.* **109**, 1355.

von Fellenberg (1930). *Biochem. Z.* **218**, 287.

First Report of the Sub-committee on the determination of arsenic, lead, and other poisonous metals in food colouring materials to the standing committee on the uniformity of analytical methods. (1930.) *Analyst*, **55**, 102.

Green (1918). *Fifth and Sixth Reports of Director of Veterinary Research, Union of S. Africa*, p. 541.

Heller (1934). *Mikrochemie*, **14**, 369.

Kleinmann and Pangritz (1927). *Biochem. Z.* **185**, 15.

Maechling and Flinn (1930). *J. Lab. Clin. Med.* **15**, 779.

Minot (1926). *J. Cancer Res.* **10**, 293.

Moir and Jamison (1925). *J. S. African Chem. Inst.* **8**, 16.

Neller (1929). *J. Assoc. Off. Agric. Chemists*, **12**, 332.

Report of the Conjoint Committee on the detection and approximate estimation of minute quantities of arsenic in beer, brewing materials, food-stuffs and fuels (1902). *Analyst*, **27**, 48.

Scott (1925). Standard methods of chemical analysis. 4th revised edition, p. 51. (Van Nostrand Co. Inc., New York.)

Youngburg and Farber (1932). *J. Lab. Clin. Med.* **17**, 363.

XCVII. THE CAROTENOIDS OF BUTTER.

By ALBERT EDWARD GILLAM and ISIDOR MORRIS HEILBRON.

From the Chemistry Department, Manchester University.

(*Received February 22nd, 1935.*)

In a series of papers [Gillam *et al.*, 1933; Watson *et al.*, 1933; 1934; Gillam, 1934] data have been collected on the carotene and vitamin A contents of the butter of several breeds of cattle fed under various conditions. In view of the fact, however, that carotene exists in more than one isomeric form [Kuhn and Lederer, 1931; Kuhn and Brockmann, 1933] and that, of these, the α- and γ-forms are physiologically only half as active as the β-isomeride [Kuhn, Brockmann *et al.*, 1933] it is obviously important to ascertain precisely in which form the pigment is present in butter. The present paper describes the results of a chromatographic examination of the carotene fractions of a number of ordinary and colostral butter samples with a view to elucidating this problem. The colostral butters, which became available during a larger experiment at present in progress, were especially useful in this investigation on account of their exceedingly high carotene content [*cf.* Dann, 1933; Semb *et al.*, 1934].

The butters (100–500 g.) were saponified as previously described [Gillam *et al.*, 1933] and the unsaponifiable material, after removal of the major part of the sterols by crystallisation from methyl alcohol, separated into carotene and xanthophyll fractions by partition between light petroleum and 90 % methyl alcohol. The methyl alcoholic solution, containing the major part of the vitamin A and all the xanthophyll, was discarded. The light petroleum solution (30–40 ml.) was washed with water to free it from methyl alcohol, dried over sodium sulphate and subjected to the Tswett chromatographic process using a 3 × 20 cm. column of alumina (Brockmann's activated Al_2O_3 (Merck) diluted 1 : 2 with inactive Al_2O_3). The chromatogram was developed by washing successively with light petroleum of B.P. 40–60° and 60–80°. The separated zones were eluted individually with light petroleum containing methyl alcohol, filtered from adsorbent and examined spectroscopically in either light petroleum or carbon disulphide.

The principal pigments.

The majority of the samples exhibited two main coloured zones which washed down the columns separately, the lower one being yellow or buff in colour whilst the upper one was brown or pink. A similar separation was observed when calcium hydroxide was employed in place of alumina [Karrer and Walker, 1933]. The spectroscopic values are shown in the accompanying table, together with the absorption maxima recorded for α- and β-carotene in the literature. As Haüsser (quoted by Kuhn, 1931) has shown that different spectroscopic techniques give slightly different values for the absorption maxima of carotene solutions, we have also made chromatographic and spectroscopic examinations of carotene samples definitely known to contain the α- and β-isomerides, under conditions exactly comparable with those used in the case of the butter pigment, employing for this purpose:

(*a*) the unsaponifiable matter of palm oil [Kuhn and Brockmann, 1931], and

(*b*) a sample of crystalline carrot carotene, M.P. 178° [Kuhn and Lederer, 1931; Mackinney, 1935].

Table I.

		Lower zone (α-carotene)			Upper zone (β-carotene)			
			Absorption maxima			Absorption maxima		
Sample	Adsorbent	Colour of zone on adsorbent	Petrol 70–80° $m\mu$	CS_2 $m\mu$	Colour of zone on adsorbent	Petrol 70–80° $m\mu$	CS_2 $m\mu$	Ratio of $\alpha:\beta$ carotene present
Ordinary butters:								
English (1)	Al_2O_3	Pink-buff	470 445	—	Red-brown	480 451	—	1:2·7
Shorthorn (2)	$Ca(OH)_2$	Brown-yellow	474 446	510 478	Red-brown	—	—	About 9:1
English Guernsey	Al_2O_3	Buff	475 447	505 479	Salmon-pink	483 453	513 482	1:2·2
Danish	Al_2O_3	Buff	473 447	—	Pink	482 452	—	1:4
New Zealand	Al_2O_3	Buff	475 447	506 477	Red	483 453	513 480	1:2·2
Colostrum butters:								
Shorthorn	Al_2O_3	Yellow	477 448	510 477	Pink-brown	481 452	—	4:1
Shorthorn	Al_2O_3	Yellow	473 446	508 477	Pink	482 452	514 481	1:4·4
Friesian	Al_2O_3	Yellow	478 448	510 478	Brown	—	—	7:1
Guernsey	$Ca(OH)_2$	Yellow	475 447	509 478	Pink-brown	480 450	—	Mainly β-form
Ayrshire	Al_2O_3	Yellow	473 446	—	Deep red	482 452	—	1:2·7
Ayrshire	Al_2O_3	Yellow	474 445	—	Salmon-pink	482 452	—	1:1·8
Carotene extracts of known composition:								
Cryst. carrot carotene M.P. 178°	Al_2O_3	Yellow	477 448	508 477	Red-brown	483 453	513 482	—
Palm oil unsaponifiable matter	Al_2O_3 (also $Ca(OH)_2$)	Bright yellow	476 447	507 475	Red-brown	481 451	513 481	—
Data on pure pigments given in the literature	$Ca(OH)_2$	Yellow	478 447·5	509 477	—	—	—	Karrer and Walker [1933]
	—	—	478 447·5	511 478	—	483·5 452	521 485·5	Kuhn and Brockmann [1932] Kuhn and Lederer [1931]
	MgO	—	—	507 476	—	—	511 485	Strain [1931]

A comparison of the data (see Table I) with the corresponding butter values definitely proves that butter contains both α- and β-carotene and that the relative amounts of these isomerides vary considerably in different samples. The ratio of the two substances present in each butter was determined as follows. The separately eluted pigments were made up to equal volumes in light petroleum B.P. 70–80°, and the intensity of absorption of light of each solution was determined at the wave length of maximum absorption (447 $m\mu$ for α-carotene, 452 $m\mu$ for β-carotene). When equal thicknesses of solution are examined the relative amounts of pigment present are directly proportional to the respective intensities of absorption recorded.

Pigments present in only small amount.

In addition to α- and β-carotene, several of the more highly pigmented butters were found to contain minor amounts of other petrol-phasic lipoid pigments, whose presence was indicated by the appearance of one or more coloured bands (usually a yellow-brown ring with a reddish violet ring just over it) situated above the β-carotene on the developed chromatogram. The yellow pigment was definitely characterised as kryptoxanthin by the following properties: (*a*) the yellow-brown colour of the ring and the fact that it is adsorbed just above the β-carotene; (*b*) absorption maxima in light petroleum at 481 and 451·5 $m\mu$. Kuhn and Grundmann [1933], using a grating spectroscope instead of our prism spectroscope, record maxima for kryptoxanthin at 485·5 and 452 $m\mu$; (*c*) the pigment, although epiphasic in light petroleum to 90 % methyl alcohol, is partially extracted by 95 % methyl alcohol; (*d*) the pigment is adsorbed only with difficulty on calcium carbonate.

In the case of one Ayrshire colostral butter the amount of this pigment was estimated to constitute approximately 3 % (*i.e.* 0·15 mg. per 100 g. fat) of the total carotene fraction.

The reddish violet band was usually present in too small quantity for definite characterisation, but in the case of the casual Danish butter sample, which was much richer than usual in both these subsidiary pigments, it was found possible to separate it almost completely from the kryptoxanthin by repeated adsorption on alumina. The pigment is characterised by absorption bands at 503, 470 and 442 $m\mu$, which agree with the maxima of lycopene recorded by Kuhn and Brockmann [1932] who give 506, 474 and 446 $m\mu$. The slight discrepancy in the wave lengths is probably due to incomplete separation of all the kryptoxanthin, and in this connection Kuhn and Grundmann have reported that these two carotenoids are not sharply separable on alumina. In this particular sample of butter, the kryptoxanthin and lycopene were present in approximately equal amounts, and together constituted nearly half of the total petrol-phasic colouring matter.

In the light of these results it was argued that as the carotenoids of milk fat must come from the food ingested by the cow *via* the blood, then the blood serum carotenoids might be expected to be similar to those of butter. A preliminary examination of the lipoid pigments of the blood serum of cows indicates a striking similarity to those of butter. We are at present examining the matter in more detail.

SUMMARY.

The petrol-phasic carotenoid fractions of the unsaponifiable matter of a number of ordinary and colostral butters have been examined by combined chromatographic and spectroscopic methods. It has been found that the carotene present is a mixture of α- and β-forms, the relative amounts of which vary considerably in different samples. Kryptoxanthin and lycopene have also been detected in small amount in several of the more pigmented samples of butter.

We wish to thank Messrs Imperial Chemical Industries Ltd. Research Council for financial assistance and to acknowledge the invaluable co-operation of Messrs Page, Watson and Ferguson of Jealotts Hill Agricultural Research Station in the collection and preparation of the butter samples.

REFERENCES.

Dann (1933). *Biochem. J.* **33**, 1998.
Gillam (1934). *Biochem. J.* **28**, 79.
—— Heilbron, Morton, Bishop and Drummond (1933). *Biochem. J.* **27**, 878.
Karrer and Walker (1933). *Helv. Chim. Acta*, **16**, 641.
Kuhn (1931). *Brit. Assoc. Report*, 108.
—— and Brockmann (1931). *Z. physiol. Chem.* **200**, 255.
—— —— (1932). *Z. physiol. Chem.* **206**, 41.
—— —— (1933). *Ber. deutsch. chem. Ges.* **66**, 407.
—— —— Scheunert and Schieblich (1933). *Z. physiol. Chem.* **221**, 129.
—— and Grundmann (1933). *Ber. deutsch. chem. Ges.* **66**, 1746.
—— and Lederer (1931). *Z. physiol. Chem.* **200**, 246.
Mackinney (1935). *J. Biol. Chem.* **108**, 45.
Semb, Baumann and Steenbock (1934). *J. Biol. Chem.* **107**, 703.
Strain (1934). *J. Biol. Chem.* **105**, 525.
Watson, Bishop, Drummond, Gillam and Heilbron (1934). *Biochem. J.* **28**, 1076.
—— Drummond, Heilbron and Morton (1933). *Emp. J. Exp. Agric.* **1**, 68.

XCVIII. STABILITY OF THE INTERNATIONAL STANDARD OF THE OESTRUS-PRODUCING HORMONE IN ALCOHOLIC SOLUTION.

By IDWAL WYN ROWLANDS and ROBERT KENNETH CALLOW.

From the National Institute for Medical Research, London, N.W. 3.

(*Received February 6th, 1935.*)

THE International Standard of the oestrus-producing hormone consists of the hydroxyketonic compound known in this country as ketohydroxyoestrin or oestrone. Since it is desirable, for some purposes, to prepare and store alcoholic solutions of the standard substance, it has been considered advisable to investigate its stability when kept in this form for various periods and at different temperatures. Butenandt [1930] reported that the stability of oestrone in alcoholic solution was small, a decrease of activity of at least one-third taking place in 2 months.

Technique.

Preparation of alcoholic solution. For biological assay a sample of the International Standard was dissolved in the proportion of 2 mg. in 100 ml. of absolute alcohol (*i.e.* 0·002 %); for spectrographic examination the concentration was 0·0125 %. Solution in the cold was slow, and was completed by heating to 50° for 10 minutes on a water-bath. The solution was cooled, transferred to dry sterile ampoules of non-alkaline resistance glass and immediately sealed.

Storage. Each set of ampoules was divided into batches. Those for biological assay were stored at $-2°$, at room temperature in the dark and at 37°, respectively. Those for spectrographic examination were divided into four batches stored (*a*) at $-2°$, (*b*) at room temperature in the dark, (*c*) at room temperature, in diffused daylight, (*d*) at 37°. Batches (*a*) and (*d*) were occasionally exposed to artificial light but otherwise kept in the dark. The absorption spectra were measured on the instrument described by Philpot and Schuster [1933].

Biological assay. The method of assay consisted in the determination, by means of a dose-response curve, of the dose necessary to cause oestrus in 50 % of a group of ovariectomised mice. It followed closely that employed by Deanesly and Parkes in some work on the suitability of the International Standard, a report of which has been submitted to the Permanent Commission on Biological Standardisation of the League of Nations Health Organisation, but not yet published.

Test animals. 111 immature albino mice about 4 weeks old were ovariectomised. After a period of about one month, control vaginal smears were taken. The mice were divided into four groups, the number in each being shown in Table I. The same group was injected with the same batch of hormone at each test, except in the last test, where four animals from group 1 were removed to group 4. A fortnight before each test all the mice were "primed" with 1γ of hormone contained in 0·5 ml. 10 % alcohol. The smears were stained in methylene blue. A mixture of nucleated and cornified epithelial cells, or cornified cells alone in the absence of leucocytes, was considered as a positive reaction.

Table I.

Number of mice per group for each test.

Group	Solution injected kept at	For standard curve	Number of animals After 3 months	 After 6 months	 After 12 months
1	37°	30	27	27	17
2	−2°	28	28	26	20
3	Room temp.	25	23	23	18
4	Control	28	24	18	18

Biological assay.

Preliminary experiment. An experiment on the freshly made alcoholic solution containing 1 mg. of crystalline hormone in 50 ml. absolute alcohol was made to verify the curve obtained previously by Deanesly and Parkes. Four aqueous solutions were made from the alcoholic solution containing known amounts of hormone as shown in Table II.

Table II.

Solution	Volume of alcoholic solution in 50 ml. H_2O ml.	Crystalline substance in 0·4 ml. of the aqueous solution γ	Percentage positive reactions
A	1·0	0·16	100
B	0·6	0·096	90
C	0·35	0·056	60
D	0·20	0·032	29

0·4 ml. of each of the solutions A–D was injected subcutaneously over 36 hours into the groups 1–4 shown in Table I. The times of injection were as follows: 1st day, 9.30 a.m., 9.30 p.m.; 2nd day, 9.30 a.m., 9.30 p.m.; and smears were taken: 3rd day, 4 p.m., 11.30 p.m.; 4th day, 9.30 a.m., 4 p.m., 11.30 p.m.; 5th day, 9.30 a.m. The percentage number of positive reactions is shown in Table II and plotted in Fig. 1.

The curve conforms in shape very closely to that obtained by Deanesly and Parkes, but it is displaced somewhat to the left so that the weight giving a 50 % response is 0·047γ (see Table III) as compared with 0·061γ for the previous curve.

Stability tests. After 3 months one ampoule from each of the batches kept at different temperatures was opened and from each 0·45 ml. of the alcoholic solution was taken and made up to 50 ml. with distilled water. 0·4 ml., of the aqueous solutions was injected over 36 hours into each mouse, which thus received 0·072γ. As shown in Table I these aqueous solutions made from the alcoholic solutions kept at 37°, −2° and room temperature were injected into groups 1, 2 and 3 respectively.

From Fig. 1 a 70–80 % response was to be expected from a fresh solution and, allowing for a slight loss in activity during the 3 months, approximately a 50 % response was aimed at. From the 100 % positive result obtained it is not possible to calculate the unit in any of the groups. This high response can only be explained by variation in the sensitivity of the mice, a fact which emphasises the need for a control group in the tests.

After 6 and 12 months the test was repeated. In both instances 0·35 ml. of the original alcoholic solution was made up to 50 ml. with water. A similar amount of the aqueous solution (0·4 ml.) was injected into the same groups of mice. The amount of hormone injected into each mouse in both of these tests was reduced to 0·056γ. After 6 months the units for the three batches and the control shown in Table III are uniformly higher than expected. The difference between the units is insignificant. The group receiving the hormone kept at 37° gave the highest response, which further shows that no loss in activity had occurred. On account of this a period of another 6 months was allowed to elapse before making a further test. After 12 months the units for the three solutions are almost identical and show very little difference from the control unit of 0·044γ. No loss in activity has occurred.

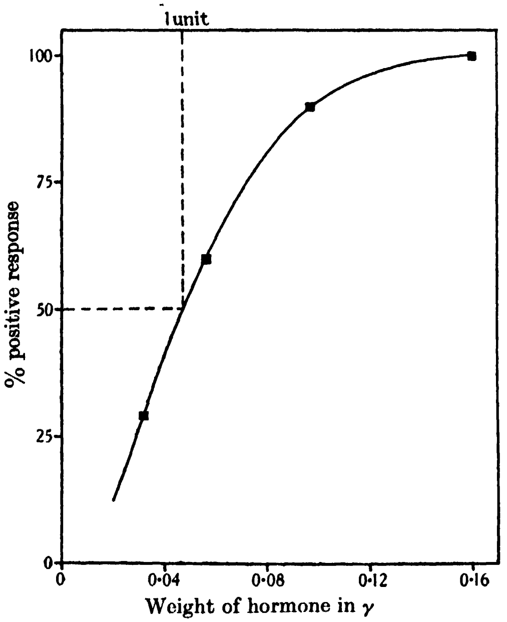

Fig. 1. International Standard of the oestrus-producing hormone; curve showing percentage positive response of vaginal smears to various doses of the hormone. The weight giving 50 % response is 0·047γ (0·47 International Unit) and is considered as one mouse unit.

Table III.

Weight of hormone in γ required to cause 50 % response.

Time from start of exp. at which the solutions were tested	Kept in alcoholic solution at			Control solution
	−2°	Room temp.	37°	
—	—	—	—	0·047
3 months	—	—	—	—
6 months	0·067	0·080	0·066	0·077
12 months	0·047	0·046	0·048	0·044

Simultaneously with each test a similar volume of a freshly made alcoholic solution (1 mg. in 50 ml. of absolute alcohol) of the International Standard was injected into group 4 so that each animal received the same amount of hormone. The results, which are shown in Table III, are expressed in terms of the amount of hormone required to induce a 50 % response (1 M.U.), the amounts being calculated from the curve in Fig. 1.

Spectrographic examination.

Fresh solutions of hormone. The absorption spectrum measured in 0·0125 % alcoholic solution was in fair agreement with that recorded by Dingemanse *et al.* [1931] showing a maximum value of $E^{1\%}_{1\,\text{cm.}}=81$ (as compared with 82·5) at 281·5$m\mu$, but the small subsidiary hump which they record at 291$m\mu$ was, in our measurements, visible only as an inflection in the curve. The curve is plotted in Fig. 2, in terms of

$$\epsilon=\frac{1}{d}\cdot\log_{10}\frac{I_0}{I}-\epsilon_0.$$

Stability measurements. Measurements were taken of the contents of one ampoule from each batch at intervals of 3, 9 and 12 months after the original sealing.

In batches (*a*), (*b*) and (*c*) no change in the absorption outside the range of the experimental error was detected. In batch (*d*) (kept at 37°) as shown in Fig. 2, there was a slight increase of doubtful significance in the absorption at the peak at 281·5$m\mu$, but at the minimum at 250$m\mu$ there was a progressive increase of absorption, amounting in all to a change in the value of ϵ from 0·06 to 0·31. This change is not sufficiently large or characteristic for definite conclusions to be drawn as to its exact cause. It may be due to decomposition of oestrone, but the retention of the shape of the absorption curve as a whole makes it extremely unlikely that, if such decomposition actually occurs, it does so to any considerable extent.

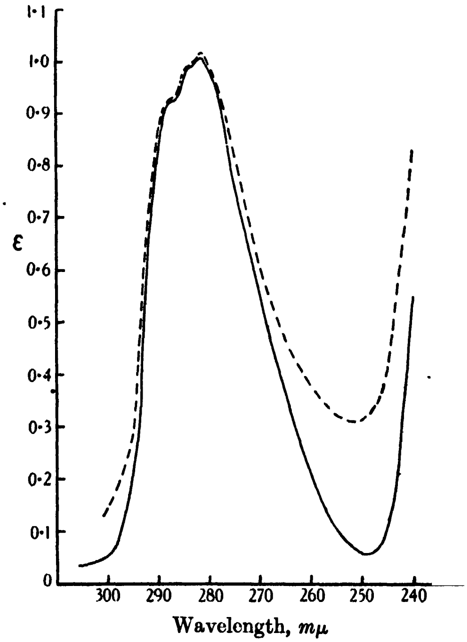

Fig. 2. Absorption spectra of solutions of the International Standard of the oestrus-producing hormone in 0·0125 % alcoholic solution.

——— Fresh solution. - - - - - Solution kept 12 months at 37°.

SUMMARY.

Alcoholic solutions of the International Standard of the oestrus-producing hormone stored in sterile sealed ampoules at −2°, room temperature or 37°, for 12 months show neither decline in activity as measured by the vaginal cornification test, nor any change in ultra-violet absorption, with the exception of a small increase in general absorption when stored at 37°.

REFERENCES.

Butenandt (1930). *Z. physiol. Chem.* **191**, 140.

Dingemanse, Kober, Reerink and van Wijk (1931). *Biochem. Z.* **240**, 265.

Philpot and Schuster (1933). *Med. Res. Coun. Sp. Rep. Ser.*, No. 177 (H.M. Stationery Office, London).

XCIX. RESEARCHES ON THE PHOSPHATASES.

II. INACTIVATION AND REACTIVATION OF THE PHOSPHATASES OF ANIMAL ORGANS.

By SERAFINO BELFANTI, ANGELO CONTARDI
and ALBERTO ERCOLI.

From the Istituto Sieroterapico Milanese and R. Istituto Superiore Agrario, Milan.

(*Received February 8th, 1935.*)

Recently Robison and Rosenheim [1934] made the interesting observation that sodium fluoride, even in extremely small quantities, impedes *in vitro* the deposition of calcium in hypertrophic cartilage; however, even in much higher degrees of concentration, it does not alter the extent of hydrolysis brought about by a purified preparation of bone phosphatase on sodium β-glycerophosphate.

In a recent paper Belfanti *et al.* [1935], after having examined the inhibitory action of sodium fluoride on the "acid" phosphatases of extracts of animal tissues, mentioned, in connection with the probable importance of the fluorides in the phenomena of calcification and decalcification of bone, the fact that in certain circumstances sodium fluoride may also inhibit the activity of the "alkaline" phosphatases.

In the present work, we have attempted to find under what conditions that phenomenon may occur.

It seemed moreover that it would be of interest to find out if the inactivation effected by sodium fluoride was permanent in character or was reversible.

Inouye [1929], when experimenting with the glycerophosphatase of *Aspergillus oryzae*, found that on eliminating the fluorine ions by the addition of an excess of calcium chloride, the phosphatase activity was not restored, whereas the inhibitory effects of oxalate ions and sulphate ions could be eliminated by the addition of $CaCl_2$.

Experimental.

As regards the preparation of the enzymic extracts and the method used to estimate the phosphatase activity, we refer the reader to our previous work and when necessary discuss any modifications that have been introduced.

Reactivation of phosphatases inactivated with sodium oxalate.

(1) *Reactivation by the addition of $CaCl_2$.* Experiments made with an extract of rabbit liver agree in the results with those made by Inouye [1929] on the phosphatase of *A. oryzae*: we obtained, in other words, the restoration of the phosphatase activity of the extract. In order to ascertain if the time of contact between the oxalate and the enzyme before treating with calcium chloride had a

sensible influence on the inactivation, a series of five numbered test-tubes each containing 7 ml. of the undermentioned solution was prepared.

Acetate-veronal solution ...	15 ml.
8·5 % solution of NaCl ...	15 „
Distilled water	12 „
Rabbit liver extract	30 „
HCl $N/5$	36 „

$M/5$ sodium oxalate solution (1 ml.) was added to test-tubes 1, 2, 3 and 4. In test-tube No. 1, this addition was made immediately, whereas in test-tube No. 2 it was made after 30 mins., in test-tube No. 3 after 85 mins. and in test-tube No. 4 after 90 mins. 1 ml. of distilled water was added to test-tube No. 5. 1 ml. of $N/2$ $CaCl_2$ solution was then added to test-tubes Nos. 1, 2, 3 and 5 whilst 1 ml. of distilled water was added to test-tube No. 4. Finally, 1 ml. of 5 % (anhydrous salt) solution of sodium glycerophosphate was added to all five test-tubes, together with two drops of toluene. The five test-tubes were then placed at the same time in a thermostat at 37° and 16 hours later the inorganic phosphorus contents were estimated. Table I shows that, even when the sodium oxalate is allowed to act for one hour and a half and at p_H less than 5 on an enzymic solution obtained from rabbit liver, there is no notable alteration in the activity of its acid phosphatase.

Table I. *Influence on the acid phosphatase of rabbit liver of the time of contact between enzymic solution and sodium oxalate before reactivation with $CaCl_2$.*

Test-tube No.	Time of contact before the addition of $CaCl_2$	p_H of the solutions under examination	P hydrolysed mg.
1	90 mins.	4·87	0·0407
2	30 „	4·91	0·0414
3	5 „	4·88	0·0402
4	Control without oxalate	4·92	0·0398
5	Control without $CaCl_2$	5·02	0·0125

(2) *Reactivation by means of dialysis.* 25 ml. of $M/10$ sodium oxalate solution (portion A) were added to 25 ml. of a rabbit liver extract, whilst another 25 ml. of the same extract were diluted with 25 ml. of distilled water (portion B). The two portions were subjected to dialysis through cellophane against distilled water for 24 hours. When the dialysis was terminated, both liquids were brought to a volume of 60 ml. with distilled water. Three 25 ml. flasks were then prepared, each of which contained: solution of sodium glycerophosphate (5 % anhydrous salt), 1 ml.; acetate-veronal solution, 2 ml.; NaCl solution, 2 ml.; N HCl, 1·5 ml. 10 ml. of the enzymic extract (A) were added to flask No. 1, 10 ml. of extract (B) were added to flask No. 2 and 10 ml. of extract (B) *plus* 5 ml. of $M/5$ sodium oxalate were added to flask No. 3. All three flasks were then filled up to the mark with distilled water. Their p_H was about 3·2. After the addition of toluene and digestion in the thermostat for 16 hours, the following results were obtained:

Flask No.		P hydrolysed per ml. mg.
1	Dialysed after the addition of sodium oxalate	0·0289
2	Dialysed without the addition of sodium oxalate	0·0277
3	Control in the presence of $M/25$ oxalate added after dialysis	0·0074

The enzymic solution inhibited with sodium oxalate had reacquired its activity during dialysis.

Reactivation of phosphatases inactivated with sodium fluoride.

(1) *Reactivation by means of addition of $CaCl_2$.* Experiments carried out both with rabbit liver extract and with rabbit kidney extract demonstrate the possibility of reactivating, at least partially, by means of addition of $CaCl_2$, an enzyme which has been previously rendered inactive with NaF. The extent of this reactivation was, however, unlike that which was found in the case of sodium oxalate, in inverse relation to the time during which the fluorine ions had acted on the enzyme of the solution before being precipitated in the form of calcium salts. The experiment was carried out in exactly the same way as those done with sodium oxalate (see Table I). Table II shows the results obtained in two experiments with rabbit liver extract and rabbit kidney extract respectively.

Table II. *Influence on the acid phosphatase of rabbit liver and of rabbit kidney of the time of contact between enzymic solution and sodium fluoride before the addition of $CaCl_2$.*

Test-tube No.	Time of contact before the addition of $CaCl_2$	p_H of the solutions under examination	P hydrolysed mg.	Reacquired activity as % of the original activity
		Rabbit liver extract		
1	90 mins.	4·94	0·0136	15·4
2	30 „	4·86	0·0286	29·1
3	5 „	4·93	0·0296	59·3
4	Control without NaF	4·86	0·0364	—
5	Control without $CaCl_2$	4·89	0·0080	—
		Rabbit kidney extract		
1	90 mins.	4·9	0·0142	37·7
2	30 „	4·9	0·0207	65·8
3	5 „	4·8	0·0226	74·0
4	Control without NaF	4·92	0·0231	—
5	Control without $CaCl_2$	5·07	0·0055	—

As may be seen from Table II, under similar conditions, the activity of the acid phosphatase of kidney extract is reacquired in a greater measure than that of the acid phosphatase of liver extract.

(2) *Reactivation by means of dialysis.* 40 ml. of rabbit liver extract were added to 8 ml. of a solution of NaF, acidified with acetic acid to p_H 4·4. The whole was allowed to stand at room temperature for three hours. Afterwards, the solution was divided into two equal parts: the first was dialysed for three days against distilled water (extract A) whilst the second was kept in the ice-chest (extract B). Simultaneously with A, 20 ml. of liver extract with the addition of 4 ml. of distilled water (extract C) were dialysed. The phosphatase activities of the three extracts as regards sodium glycerophosphate and in the presence of veronal buffer were then estimated in the usual way.

An experiment was also carried out, by the same method, on an extract of hog kidney prepared according to Asakawa [1929].

The following results were obtained:

Extract		Rabbit kidney		Hog kidney	
		p_H of the solution	P hydrolysed per ml. mg.	p_H of the solutions	P hydrolysed per ml. mg.
A	Dialysed after adding NaF	5·4	0·0243	4·77	0·0818
B	Not dialysed after adding NaF	5·2	0·0040	4·60	0·0225
C	Dialysed without adding NaF	5·4	0·0389	4·72	0·0803

These experiments show that dialysis leads to the partial reactivation of the acid phosphatases of extracts of rabbit and hog kidney previously inactivated with sodium fluoride. We wish to point out, however, that the contact between fluoride and enzyme, although protracted over a very long period of time, took place at room temperature instead of at 37° as was the case in the experiments on reactivation with calcium chloride.

Inactivation with sodium fluoride of the "alkaline" phosphatase of the liver and kidney. Lability of bone phosphatase in an acid medium.

In each of four 25 ml. flasks, numbered from 1 to 4, were placed:

	No. 1 ml.	No. 2 ml.	No. 3 ml.	No. 4 ml.
Liver extract	5	5	5	5
Acetate-veronal solution	4·5	4·5	4·5	4·5
N/5 HCl	6	6	—	—
2 % NaF	1	—	1	—
H_2O	—	1	6	7

The four flasks were left in the water-bath at 37° for 2 hours and then to each of them were added:

N/5 NaOH	5·5	5·5	—	—
5 % glycerophosphate	1	1	1	1

and the whole brought up to the mark with distilled water. After determination of the p_H, addition of toluene and digestion in the thermostat for 16 hours, we proceeded to estimate the P hydrolysed. The following is the result of the four tests:

No. 1	p_H 8·65	P hydrolysed per ml. mg. 0·0235
No. 2	p_H 8·80	,, ,, 0·0484
No. 3	p_H 8·62	,, ,, 0·0605
No. 4	p_H 8·90	,, ,, 0·0610

A second experiment, carried out with another extract of liver of the rabbit, much more active than the previous one, gave the following results:

No. 1	p_H 9·27	P hydrolysed per ml. mg. 0·1000
No. 2	p_H 9·32	,, ,, 0·1935
No. 3	p_H 8·70	,, ,, 0·1818

The data given above show that exposure to 37° for two hours at p_H about 4·5 partially inactivates the "alkaline" phosphatase of rabbit liver extract. In presence of sodium fluoride, the resultant inactivation is much higher.

The alkaline phosphatase of the kidney behaves in an analogous manner, as is shown by the results of an experiment made with an extract of hog kidney prepared according to Asakawa's method [1929], in which exposure to 18° in an acid medium at p_H 4·5 was protracted for 17 hours.

No. 1	p_H 8·71	P hydrolysed per ml. mg. 0·0182
No. 2	p_H 8·75	,, ,, 0·1066
No. 3	p_H 8·85	,, ,, 0·2286

Similar experiments were carried out with an extract of bone (metaphysis and epiphysis) from the adult rabbit and the results obtained were as follows:

No. 1	p_H 8·65	P hydrolysed per ml. mg. Traces
No. 2	p_H 8·70	,, ,, Traces
No. 3	p_H 8·95	,, ,, 0·0631
No. 4	p_H 8·90	,, ,, 0·0727

As exposure for two hours to 37° in an acid medium had of itself completely inactivated the alkaline phosphatase, the experiment was repeated for shorter periods of time. In a second test at p_{H} 4·5 the mixture was heated for only 20 mins.:

No. 1	p_{H} 8·66	P hydrolysed per ml. mg.	Traces
No. 2	p_{H} 8·67	,, ,,	Traces
No. 3	p_{H} 9·06	,, ,,	0·0789
No. 4	p_{H} 9·05	,, ,,	0·0789

In a third experiment the mixture was heated for 5 mins. at p_{H} 4·5 and at 37°:

No. 1	p_{H} 8·83	P hydrolysed per ml. mg.	0·0125
No. 2	p_{H} 8·80	,, ,,	0·0130
No. 3	p_{H} 8·84	,, ,,	0·0670
No. 4	p_{H} 8·84	,, ,,	0·0720

These experiments show that the "alkaline" phosphatases may be inactivated by sodium fluoride when the latter acts for a certain period of time in an acid medium. The results also show that the alkaline phosphatase of bone is much more sensitive to the inactivating action of the acid medium than the alkaline phosphatases of the liver and of the kidney.

SUMMARY.

1. The "acid" phosphatases of extracts of liver and of kidney are reversibly inactivated by sodium oxalate and can be reactivated without loss by precipitating the oxalate ions by means of calcium salts or removing them by dialysis.

2. Inactivation of the "acid" phosphatases of the liver and of the kidney by sodium fluoride is of a different nature from that produced by oxalate. In the latter case, there is probably a formation of an inactive enzyme-oxalate complex which can easily be split up, restoring the unaltered enzyme. The formation of the inactive enzyme-fluoride complex on the other hand is accompanied by a profound alteration of the enzyme, which occurs in a relatively long period of time and may lead to the permanent disappearance of the phosphatase activity. Eliminating the fluorine ions from the system more or less rapidly restores the enzymic activity in a greater or less degree. Permanent inactivation of the "acid" phosphatase by sodium fluoride occurs more rapidly at 37° than at room temperature.

3. If the "alkaline" phosphatases of the extracts of kidney and of liver at 37° are kept at p_{H} about 4·5 and then brought back to an alkaline p_{H}, there occurs an inactivation of greater or less degree according to the duration of the treatment in an acid medium.

4. Sodium fluoride also inactivates the "alkaline" phosphatases of extracts of liver and of kidney when it is allowed to act for some time on these extracts brought to p_{H} about 4·5. This is probably due to the fact that the formation of the inactive fluoride-alkaline phosphatase complex occurs only in an acid medium. By this process the inactivation of the alkaline phosphatase occurs in a much more rapid and complete manner than occurs with a simple acid treatment.

5. The "alkaline" phosphatase of bone is extremely sensitive to the acid reaction of the medium and in this is different from the "alkaline" phosphatase of the liver and of the kidney as the latter is much less sensitive. This provides evidence that the "alkaline" phosphatase of the liver and of the kidney is not identical with that of bone, a subject which will be further discussed in our next paper.

REFERENCES.

Asakawa (1929). *J. Biochem.* **10**, 157.
Belfanti, Contardi and Ercoli (1935). *Biochem. J.* **29**, 517.
Inouye (1929). *J. Biochem.* **10**, 406.
Robison and Rosenheim (1934). *Biochem. J.* **28**, 689.

C. FAT METABOLISM IN FISHES.

VI. THE FATS OF SOME PLANKTON CRUSTACEA.

By JOHN ARNOLD LOVERN.

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research.

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In previous papers [Lovern, 1932; 1934, 2] it has been shown that the fats from freshwater fish, taken as a whole, form a class distinct from those of marine species. It was suggested that differences of food might account for this, but at that time no data were available concerning the type of fat ingested in each case. The larger carnivorous fish (including all the marine species examined and all but one of those from fresh water) consume smaller creatures, mainly other fish in most cases, and here presumably the fat is of a type similar to that of the larger fish. The very small fish live largely on copepods and similar small crustacea, which are, indeed, the main link between vegetable and animal life in both salt and fresh water. The planktonic crustacea are almost exclusively vegetable feeders. It is of interest to know whether the same class distinction holds between freshwater and marine zooplankton as obtains for the fishes. If it does the idea of food differences being the cause of the observed distinctions in the case of the larger fish is considerably strengthened. This theory will receive further support if the composition of the zooplankton fats is closely similar to that of the fish fats, suggesting that ingested fat is largely deposited unchanged.

Very little is known regarding the composition of these crustacean fats. Collin *et al.* [1934] examined the fat from *Calanus finmarchicus* and also mixed copepod fat. Their results, whilst not giving details of composition, indicated a resemblance of type between the fatty acids of these fats and those of fish fats. They further found appreciable quantities of wax alcohols present. No freshwater crustacea seem to have been examined.

In this paper the results obtained with the fats from one marine species and three freshwater species are given.

EXPERIMENTAL.

The fats were obtained by desiccating with anhydrous sodium sulphate and extracting with ether. The marine species used was *Calanus finmarchicus*. This is a relatively large organism and easily obtained pure by the use of tow nets of a suitable mesh. It was collected in large quantities in Loch Sunart, Argyllshire, during May 1933, and sufficient fat was available for a full-scale quantitative analysis (200 g.).

As for freshwater organisms, it is often the case that a particular pond or lake is dominated by one organism, and by the use of a suitable net fairly pure samples may be collected. From a small sheet of water near Aberdeen, a considerable quantity of *Cyclops strenuus* was obtained almost pure, the contaminants being mainly other species of *Cyclops*. From Loch Lochy, Inverness-shire, a pure sample of *Daphnia galeata* was obtained and from Loch Ness a somewhat mixed sample, almost all of the genus *Diaptomus* with *D. gracilis* greatly predominating. There will be no serious error in considering this as all *D. gracilis*. From these freshwater organisms only sufficient fat was available in each case for analysis by semi-micro-methods [Lovern, 1934, 1]. All the fats were deep red in colour and all contained a considerable quantity of acid pigment matter. The characteristics of the fats are given in Table I, and the compositions of the mixed fatty acids in Table II, as weight and molar percentages.

Table I. *Characteristics of fats.*

Species	Saponification equivalent	Iodine value	% saponifiable
Calanus finmarchicus	457·5	177·6	32·0
Cyclops strenuus	341·7	236·2	20·0
Daphnia galeata	410·7	142·9	22·3
Diaptomus gracilis	367·6	153·8	17·5

Table II. *Compositions of mixed fatty acids.*

	Saturated acids				Unsaturated acids				
Species	C_{12}	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
					Weights %				
C. finmarchicus	—	8·3	10·6	1·3	1·6 (−2·0H)	11·8 (−2·4H)	16·8 (−5·1H)	24·5 (−7·8H)	25·1 (−8·1H)
C. strenuus	—	5·9	15·6	1·0	2·9 (> −2·7H)	30·3 (−3·0H)	25·2 (−5·2H)	15·6 (−8·6H)	3·5 (−?H)
D. galeata	0·9	3·5	13·0	1·7	2·8 (> −2·0H)	21·1 (> −2·0H)	44·9 (−5·0H)	12·1 (−8·0H)	Nil
D. gracilis	—	2·6	20·0	1·6	Nil	15·7 (> −2·0H)	34·6 (−4·5H)	25·5 (−7·1H)	Nil
					Mols. %				
C. finmarchicus	—	10·3	11·7	1·3	2·0	13·2	17·1	22·9	21·5
C. strenuus	—	6·9	16·3	0·9	3·4	32·0	24·1	13·7	2·7
D. galeata	1·2	4·1	13·6	1·6	3·3	22·3	43·2	10·7	Nil
D. gracilis	—	3·1	21·4	1·6	Nil	16·9	34·0	23·0	Nil

DISCUSSION.

It will be observed that all the fats contain large amounts of unsaponifiable matter. Whilst this was not examined in detail, its appearance strongly suggested the presence of wax alcohols, in agreement with the finding of Collin *et al.* [1934].

It will be seen from Table II that all the fats contain the typical "fish oil" fatty acids (palmitoleic, C_{20} acids *etc.*). Moreover, the class distinction between fats of marine and freshwater origin is markedly in evidence. This difference has been previously defined [Lovern, 1932] as increased proportions of C_{16} and C_{18} unsaturated acids and decreased C_{20} and especially C_{22} acids in the freshwater fats, as contrasted with those from marine sources. *D. gracilis* fat is the least typically freshwater fat, but even here the entire lack of C_{22} acids and the high content of C_{18} unsaturated acids would put this fat into the freshwater class.

A point of interest is the extremely high average unsaturation of the C_{18} and C_{20} unsaturated acids, as well as the presence of polyethylenic C_{16} acids in all cases.

It would seem that the fats of the vegetable-feeding zooplankton, from both salt and fresh water, correspond so closely to those of the marine and freshwater fish respectively that a simple deposition of ingested fat, largely unchanged in composition, would account for the observed characteristics of marine and freshwater fish fats. It may well be that the whole character of fish fats is due to the type of fat occurring in their essential food supply—the plankton crustacea.

It would be of interest to know whether the compositions of the crustacean fats are in turn largely decided by the type of fat in the food; whether, in fact, as suggested previously [Lovern, 1932], it is really the plant life of the medium which is affected by the presence or absence of salt. Experiments in progress point to this being the case and suggest that even in the crustacea the ingested vegetable fat is mainly deposited unchanged.

SUMMARY.

The fats from three freshwater and one marine species of plankton crustacea have been examined. The mixed fatty acids of the freshwater species show the same class distinction when contrasted with the acids of the marine species as is observed with the fatty acids of fish from the respective habitats.

The close correspondence in type of the fats from crustacea and fish suggest that fat ingested by the fish is largely deposited unchanged, and that the class differences observed between the fats from freshwater and marine fish as a whole have a dietary origin.

The author desires to express his thanks to Dr S. G. Gibbons and Miss H. S. Ogilvie, of the Marine Laboratory of the Fishery Board for Scotland, Aberdeen, for kindly examining the various hauls of plankton.

REFERENCES.

Collin, Drummond, Hilditch and Gunther (1934). *J. Exp. Biol.* **11**, 198.
Lovern (1932). *Biochem. J.* **26**, 1978.
—— (1934, 1). *Biochem. J.* **28**, 394.
—— (1934, 2). *Biochem. J.* **28**, 1961.

CI. *POST MORTEM* BREAKDOWN OF GLYCOGEN AND ACCUMULATION OF LACTIC ACID IN FISH-MUSCLE AT LOW TEMPERATURES.

By JAMES GARDEN SHARP.

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research.

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The temperature of maximum rate of change.

In a previous publication [Sharp, 1934] it was shown that the rate of glycogenolysis in fish-muscle after death decreased with temperature down to 0°. Lowering of temperature to −10° resulted first of all in an increased rate of change in the frozen tissue at −2° and secondly in a falling off to a barely perceptible rate at −10°. It must follow therefore that between 0° and −10° there is a temperature at which the maximum rate of breakdown occurs in the frozen muscle.

The first part of the present work shows that the maximum rate of change takes place in the temperature interval −3·2° to −3·7°. The sampling error of the technique previously given in detail is too large to permit of more accurate determination.

Experimental.

The method of experiment was slightly different from that employed previously, for no attempt was made to obtain complete curves over any period of storage. The muscle was stored for an arbitrary period at different temperatures and the loss in glycogen and the gain in lactic acid were estimated over this period. Using ten similar fresh samples from five haddocks, two were retained for immediate analysis and the remainder stored in pairs in air-tight

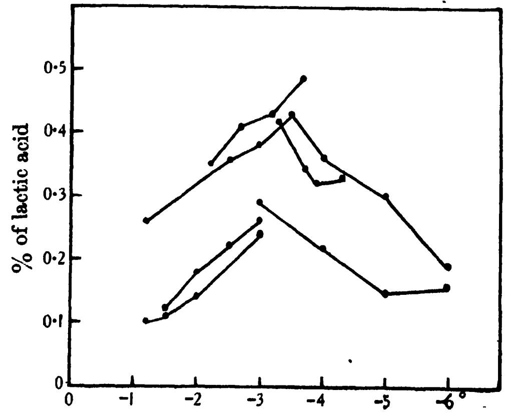

Fig. 1. Amounts of lactic acid accumulation in six lots of mixed muscle samples over arbitrary periods of 9–12 hours' storage at different freezing temperatures.

tinned containers at four temperatures for a definite period (9–12 hours) after which glycogen and lactic acid estimations were carried out by the methods given before in detail. Duplicate samples at each temperature served to reduce the sampling error and in most cases repeat experiments were done on different batches of fish. The results of six experiments are plotted in Fig. 1, and since in all cases the glycogen loss was accounted for by a corresponding increase in lactic acid, the plotted values represent either lactic acid accumulation or glycogen loss over the period of storage.

Conclusion.

In the temperature interval −3·2° to −3·7° the maximum rate of glycogen breakdown with corresponding lactic acid accumulation occurs in the frozen muscle.

In frog-muscle, Smith and Moran [1930; Smith, 1931] have shown that the increased rate of change below −0·9° is not due to oxygen starvation, but can be directly related to the amount of available water withdrawn from the muscle plasma in the form of ice. Freezing of the tissue causes increased salt concentration in the plasma, and, on the assumption that the plasma behaves like an isotonic solution of sodium chloride, it is estimated that at −2·5°, 80–82 % of the available water is withdrawn from the plasma in the form of ice, resulting in a five-fold increase in salt concentration.

In minced frog-muscle suspended in phosphate solutions of different concentrations maximum formation of lactic acid occurs in solutions freezing at −3°. Further, the solubility of rabbit-muscle globulin reaches a maximum in phosphate solutions freezing at −2° and falls rapidly in solutions freezing below −2° [Smith, 1932].

It seems therefore that the maximum rate of change takes place in the frozen muscle when a definite salt concentration is attained in the plasma, coincident with maximum solubility of the muscle globulin. Further increase in salt concentration by lowering of temperature results in decreasing solubility of the protein and decreasing rate of change.

The salt concentration of fish-muscle plasma is such that the freezing-point is approximately 0·6° lower than that of frog-muscle plasma. It follows from this that the plasma salt concentration attained in fish-muscle frozen at −3·2° would approximate to the concentration attained in frog-muscle at about −2·5°. Considered from the standpoint of the maximum rate of change occurring on the attainment of a definite salt concentration in the plasma, the present results agree closely with those obtained in the case of frog-muscle.

The critical temperature of freezing.

In frog-muscle with certain degrees of freezing, irreversible changes take place in the colloidal structure of the tissue. That such changes occur is evident on thawing the frozen muscle when it is observed that the muscle has become non-irritable and has lost the power of synthesising both glycogen and phosphagen [Moran, 1929; Smith, 1929; Kreps, 1931]. According to the intensity of freezing, muscles on thawing either return completely to normal condition or are irretrievably altered. For the gastrocnemius of the frog, these irreversible changes occur on freezing at −1·6° and storing at this temperature for a period of 24 hours. For example, on thawing at 5° muscles which have undergone different degrees of freezing, it is found that in the muscles stored at temperatures above −1·6° for 24 hours the accumulated lactic acid is resynthesised to glycogen until the normal resting level (0·05 %) is attained, whereas in muscles stored at −1·6° and lower temperatures, lactic acid continues to accumulate at a rapid rate until the rigor maximum is reached. In the latter case the synthetic mechanism for the formation of glycogen from lactic acid is irreparably damaged.

Since, in fish-muscle, there is no resting level but a comparatively rapid accumulation of lactic acid at 0° and higher temperatures, both the proof of the existence and the determination of a critical temperature of freezing present greater difficulty than in the case of frog-muscle. Experimental proof depends on comparison of the rates of formation of lactic acid on thawing muscle which has suffered different degrees of freezing. The rate of acid accumulation at temperatures in the region −2° to −4° is however so great that only short periods of storage are permissible, since an appreciable concentration of glycogen is necessary in the muscle, just before thawing, before any true comparison of rates can be made.

Experimental.

That freezing at −20° damages the lactic acid recovery mechanism in fish-muscle is evident by the results plotted in Fig. 2. Of ten similar mixed samples of muscle, five were frozen and stored separately at each of the chosen temperatures, +5°, 0°, −2·5°, −3° and −4°. The remaining samples were rapidly frozen at −20°, immediately raised to their respective storage temperatures and kept thereat, with the duplicate samples, for a period of 10 hours, after which glycogen and lactic acid concentrations were estimated. Thus, at each temperature there were two samples, one of which had been rapidly frozen to

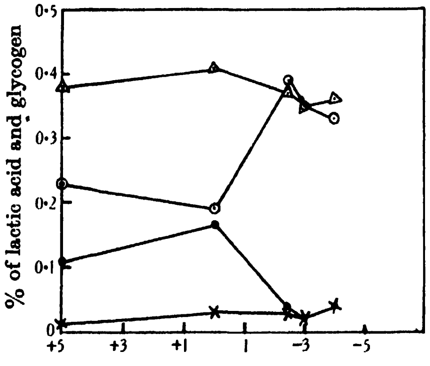

Fig. 2.

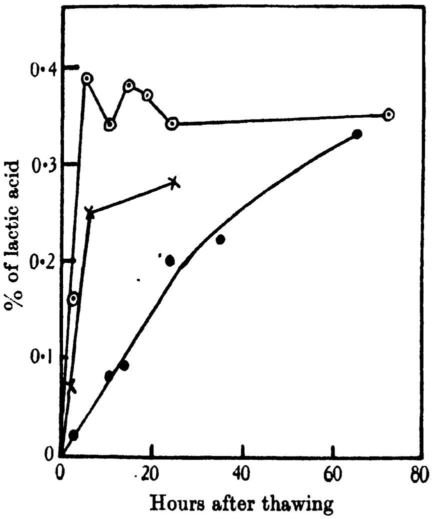

Fig. 3.

Fig. 2. Lactic acid and glycogen concentrations in mixed samples of muscle after storage for 10 hours at different temperatures.

△ Lactic acid in samples frozen at −20°. ⊙ Lactic acid in samples stored directly at temperature.
× Glycogen „ „ „ ● Glycogen „ „ „ „

Fig. 3. Rates of lactic acid accumulation at 4° in muscle, thawed after a freezing period of 4 hours at −1·6°, −2° and −6°.

●—● −1·6°. ×—× −2°. ⊙—⊙ −6°.

−20° and immediately raised to the temperature of storage, while the other had never been subjected to temperatures below the storage temperature.

The middle curves illustrate the different rates of change in the temperature interval +5° to −4°, showing maximum change between −2·5° and −4°, but, owing to an almost total loss of glycogen in this region over the storage period, the peaks of the curves have become flattened out. The effect of preliminary freezing at −20° is apparent at 0° and +5°, at which temperatures total loss of glycogen and maximum accumulation of lactic acid have occurred in the frozen muscle samples. Since only a fraction of this difference can be accounted for by the rapid double passage through the interval where maximum rate of change occurs, it appears that in freezing at −20° the synthetic mechanism in the lactic acid-glycogen cycle has been destroyed, resulting in an increased rate of lactic acid accumulation in the thawed muscle.

It remained to be determined what degree of freezing was necessary to damage this synthetic mechanism in the muscle, and a comparison was made of the rates of lactic acid formation in thawed muscle which had undergone different degrees of freezing.

Similar lots of samples were frozen at −1·6°, −2° and −6°, and after a period of 4 hours at the freezing temperature were thawed in circulating brine at 4°. Samples were removed at intervals for lactic acid estimation. The rates of lactic acid accumulation in the thawed muscle are shown in Fig. 3, the moment at the freezing temperature just before thawing being taken as zero time. On thawing muscle which has been frozen at −1·6° the normal rate of change at 4° is resumed, whereas on thawing muscle which has been frozen at either −2° or −6° a very rapid rate of change takes place, until the lactic acid maximum is attained. Between −1·6° and −2° therefore a critical temperature of freezing similar to that obtained in the case of frog-muscle must exist, for, in freezing below this temperature the muscle is irretrievably altered, in so far as the recovery mechanism for the removal of accumulated lactic acid is broken down.

SUMMARY.

1. In fish-muscle in the frozen state the maximum rate of glycogenolysis occurs in the interval −3·2° to −3·7°.

2. Freezing at −2° and lower temperatures for a period of 4 hours causes injury to the muscle, resulting in very rapid lactic acid formation on thawing. Freezing at −1·6° has no such effect, and on thawing the normal rate of lactic acid formation is resumed. Between −1·6° and −2° a "critical" temperature of freezing exists.

REFERENCES.

Kreps (1931). *Proc. Roy. Soc. Lond.* B **108**, 545.
Moran (1929). *Proc. Roy. Soc. Lond.* B **105**, 177.
Sharp (1934). *Proc. Roy. Soc. Lond.* B **114**, 506.
Smith (1929). *Proc. Roy. Soc. Lond.* B **105**, 198.
—— (1931). *Proc. Roy. Soc. Lond.* B **108**, 553.
—— (1932). *Food Investigation Board Rep.* p. 26.
—— and Moran (1930). *Proc. Roy. Soc. Lond.* B **106**, 122.

CII. GLYCOGENOLYSIS IN FISH-LIVER AT LOW TEMPERATURES.

By JAMES GARDEN SHARP.

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research.

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Since the time of Claude Bernard it has been known that the end-products of glycogenolysis in muscle and liver are different, but whilst considerable information is now available about this process in muscle, comparatively little is known regarding the detailed mechanism of glycogenolysis in liver. Results obtained from study of the breakdown of glycogen and accumulation of lactic acid in fish-muscle at low temperatures [Sharp, 1934 and preceding paper] made it of additional interest to enquire into the *post mortem* glycogenolytic changes taking place in fish-liver under similar conditions of freezing and storage.

As in the previous work, haddocks which had been lightly hooked on lines set off-shore were put into tanks of circulating sea water and kept there until, after 7–10 days' captivity, they returned to a normal resting condition. When required for experiment the fish were netted quickly, killed by a blow on the back of the head and the livers excised immediately. In order to exclude initial changes due to the struggling of the fish during catching, only fish which had undergone a minimum of struggling were used for experiment. Preliminary work was concerned with the distribution of carbohydrate throughout the tissue, immediately *post mortem*, and with the development of a reliable technique for estimation of the free sugar fraction.

Experimental.

Methods.

Glycogen concentrations were determined by Pflüger's method, the reducing values of the final hydrolysates being estimated by the Shaffer-Hartmann reagent. In the tables all glycogen concentrations are given in glucose equivalents.

For estimation of lactic acid the tissue was extracted with ice-cold 5 % trichloroacetic acid and the interfering substances were removed from the filtrate by copper-lime precipitation, according to Holmes and Gerard [1929]. The final analyses were carried out by the method of Friedemann *et al.* [1927].

Free sugar was extracted with ice-cold alcohol and the fraction freed from protein by the technique to be described later under method A.

Distribution of carbohydrate in liver tissue.

Each liver after excision was pressed gently between filter-papers to remove adherent blood and then sliced into 2, 4 or 6 samples. The samples were immediately introduced into tared receptacles containing the necessary extractants. The results, given in Table I, show only slight differences in the distributions of glycogen, free sugar and lactic acid throughout the tissue.

Table I. *Distribution of glycogen, free sugar and lactic acid in fish-liver tissue.*

Fish	Sample	Glycogen %	Fish	Sample	Glycogen %
A	1	1·02	C	1	3·68
	2	1·27		2	3·50
	3	1·04		3	3·66
	4	1·03		4	3·72
B	1	2·54	D	1	2·85
	2	2·33		2	2·85
	3	2·42			
	4	2·39			
		Free sugar %			Lactic acid %
E	1	0·024	E	4	0·030
	2	0·024		5	0·031
	3	0·023		6	0·025

Obvious differences in the concentration of glycogen between separate livers are to be noted however, and in Table II are given the glycogen and lactic acid concentrations in the livers of seven individual haddocks. The lactic acid values do not show large individual variations and lie within the range 0·015–0·031 %. On the other hand, as previously noted, large individual variations are evident in the glycogen concentrations, the values ranging from 0·12 to 3·72 % in Tables I and II.

Table II. *Glycogen and lactic acid concentrations in seven individual fish using the whole liver for estimation.*

Fish ...	1	2	3	4	5	6	7
Glycogen (%)	0·94	0·23	1·24	0·12	0·12	0·57	1·70
Lactic acid (%)	0·031	0·02	0·02	0·015	0·015	0·02	0·02

These estimations were done in the month of May, with fish caught after the spawning season and not in very healthy condition. Although the data are too few for statistical treatment, it appears that at this season of the year very low glycogen values are to be associated with "spent" fish and the higher values with immature, "unspent" fish.

Free sugar estimation.

Before following the changes in glycogen, free sugar and lactic acid concentrations taking place during storage periods at different temperatures, test experiments were made on methods of extraction and purification of the sugar fraction. Since the concentration of sugar in the liver immediately after excision is rather low for test purposes, a higher range of values was obtained by allowing glycogenolysis to proceed for different periods before analysis of the tissue.

Results are given in Table III, the details of the several experimental methods being as follows:

A. The minced sample was stirred up in 10 volumes of alcohol of 90 % effective concentration and left to extract overnight at room temperature. The extract, after being made to known volume next morning, was filtered and an aliquot sample freed from alcohol by evaporation in a current of hot air. The residue was taken up in water and one-fifth volume of "colloidal iron" and one-tenth volume of saturated sodium sulphate were added to precipitate protein. The reducing power of the final solution was estimated by the Shaffer-Hartmann reagent.

B. The tissue was extracted three times with successive 10 volumes of 90 % alcohol, after which the collected filtrates and washings were made to known volume and an aliquot sample treated as in A.

C. To the final solutions obtained by either A or B method, the copper-lime technique of Holmes and Gerard [1929] was applied as a further precaution against the presence of non-carbohydrate reducing substances.

D. After evaporation of the alcoholic extract and solution of the residue in water an aliquot sample was hydrolysed in 2·2 % hydrochloric acid for 3 hours at 100° and after neutralisation was treated with "colloidal iron" and saturated sodium sulphate as in A.

Table III. *Estimation of free sugar in minced liver tissue by different methods.*

Liver sample	% free sugar by method A	B	C	D
1	0·24	0·25	0·27	0·27
2	0·45	0·43	0·46	0·49
3	0·45	—	—	0·47
4	0·56	0·56	—	—
5	0·60	0·59	0·59	0·67

The results demonstrated that there were no appreciable differences in the sugar values obtained by methods A, B and C, and the negligible increases in reducing values, obtained after acid hydrolysis, proved that no polysaccharides were being extracted.

In the work now to be described, as in the distribution experiments, method A (above) was adopted and the reduction values, so obtained, were further checked by Somogyi's [1928] yeast fermentation technique.

Post mortem *glycogenolysis at 0°.*

The livers excised from 9–12 haddocks immediately after netting and stunning were minced and thoroughly mixed together into a uniform paste in a cold room at 0°. Samples were stored separately in air-tight tins in a brine bath thermostatically controlled to remain at 0° ± 0·03°, and single samples removed at intervals for estimation of glycogen, free sugar and lactic acid. The results of one representative experiment are plotted in Fig. 1.

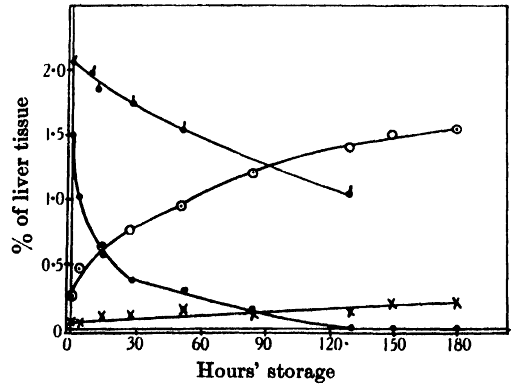

Fig. 1. Changes occurring in glycogen, free sugar and lactic acid concentrations during storage of minced liver tissue at 0°. Glycogen •—•; free sugar ⊙—⊙; lactic acid x — x; glycogen at −3° ♠—♠.

The rate of sugar accumulation was lower in all cases than the rate of glycogen breakdown, but whereas the rate of glycogen loss steadily diminished with depletion of the stores, free sugar continued to accumulate steadily until a balance was obtained between glycogen loss and gain in sugar and lactic acid.

The lactic acid concentration, although increasing steadily, remained at a comparatively low level throughout. In this particular experiment a total glycogen loss of 1·5 % of the liver was balanced by a sugar gain of 1·31 % *plus* a lactic acid gain of 0·13 %.

A comparison was made of the rates of change in oxygen and nitrogen. The samples were stored in gas-filled desiccators in a cold chamber at 0°. The results obtained (Table IV) proved that the reactions were anaerobic, similar rates of change occurring in the two gases.

Table IV. *Comparison of changes occurring in minced liver tissue during storage in oxygen and in nitrogen.*

	Stored in oxygen			Stored in nitrogen		
Hours' storage at 0°	Glycogen %	Lactic acid %	Fermentable free sugar %	Glycogen %	Lactic acid %	Fermentable free sugar %
20 mins. *post mortem*	4·50	0·03	0·04	4·50	0·03	0·04
7	4·05	0·03	0·15	4·20	0·04	0·15
12	3·76	0·03	0·18	3·85	0·04	0·19
24	—	0·03	0·30	—	0·04	0·30
55	3·18	0·07	0·54	3·20	0·07	0·54
99	2·34	0·07	0·62	2·34	0·09	0·58
169	1·33	0·20	1·14	—	0·20	1·06
247	0·95	0·13	1·30	0·90	0·18	1·20
409	0·14	0·14	1·43	0·16	0·16	1·37

With the very high initial concentration of glycogen experienced in this case, the lag in sugar accumulation was very pronounced, since after 409 hours' storage there was still a glycogen loss of approximately 3·0 % of the liver which could not be accounted for in accumulation of either free sugar or lactic acid.

From these results it appears that during glycogenolysis there is rapid accumulation of some intermediary substance which in its turn breaks down at a comparatively slow rate to form free sugar.

In a further experiment the changes in the total carbohydrate fraction were followed in addition to the changes in glycogen and free sugar. West, Scharles and Peterson's [1929] method for total carbohydrate estimation was employed. The liver tissue after hydrolysis in *N* H_2SO_4 was treated with mercuric sulphate to precipitate protein, the reducing power of the filtrate estimated by Shaffer-Hartmann reagent and the values so obtained checked by yeast fermentation. The method was tested by addition of pure glucose to several hydrolysates and in all cases 99–100 % recovery was obtained.

The changes occurring in the various fractions at 0° are given in Table V.

Table V. *Changes occurring in glycogen, free sugar and total carbohydrate concentrations during storage of minced fish-liver tissue at 0°.*

Hours' storage at 0°	Glycogen (G) %	Free sugar (S) %	Total carbohydrate (T) %	Sum $G+S$	Δ $T-(G+S)$
20 mins. *post mortem*	2·66	0·06	2·86	2·72	0·14
50	1·38	0·50	2·83	1·88	0·95
98	0·42	0·64	2·75	1·06	1·69
175	0·08	1·05	2·88	1·13	1·75
314	Trace	1·31	2·60	1·31	1·29
508	Nil	2·03	2·50	2·03	0·47

If the difference between total carbohydrate and the sum of the glycogen and sugar values be taken as a measure of the unknown intermediary fraction it is seen that this fraction was present initially only to the extent of 0·14 % of the tissue. It accumulated at a rate intermediate between the rates of breakdown of glycogen and accumulation of free sugar to reach a maximum when only 0·08 % glycogen remained in the tissue and thereafter it decreased steadily with the continued accumulation of free sugar after the glycogen store had become exhausted.

In mammalian liver similar evidence for the accumulation of an intermediary substance was brought forward by Simpson and Macleod [1927], and more recent work by Barrenscheen *et al.* [1930] indicated that *post mortem* glycogenolysis during the first 4 hours proceeded in a step-like fashion, periods of rapid change in sugar, inorganic phosphate and hexosemonophosphate concentrations being followed by periods of slower rates of change. The periods of increased hexose ester accumulation however were coincident with the periods of decreased formation of sugar and inorganic phosphate, which suggested that alternate synthesis and degradation of hexose ester occurred.

Although the plotted values obtained in the present work provide smooth curves, the possibility of a step-by-step process of glycogenolysis occurring in fish-liver is not precluded. The chosen periods of sampling were of such duration that a possible step-by-step formation might well be completely masked. The position of hexosemonophosphate in the glycogenolytic processes of fish-liver and the identification of the intermediate fraction are points which await further study.

The effect of freezing on the rate of glycogenolysis.

It is known that certain degrees of freezing bring about increased rates of glycogenolysis in fish-muscle. With more intense freezing decreased rates of change occur until at −10° very small changes are evident over long periods [Sharp, 1934].

In the liver the same conditions do not exist and lowering of temperature to below the freezing-point results in a continually decreasing rate of change in the frozen tissue. The curve of glycogen breakdown at −3°, introduced for comparison in Fig. 1, shows that the rate of change at 0° is almost three times as great as at −3°. Similar decreases are observed in the rates of accumulation of sugar and lactic acid.

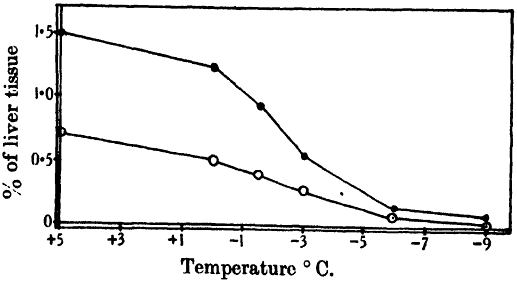

Fig. 2. Relative amounts of glycogen loss and sugar accumulation in minced liver tissue over a period of 53 hours at different temperatures. Glycogen values •; free sugar values ⊙.

In Fig. 2 are plotted the amounts of glycogen breakdown and sugar accumulation over a period of 53 hours at temperatures in the interval +5° to −9°. It is seen that the rates of glycogen breakdown and sugar accumulation decrease markedly through freezing the tissue to $-1{\cdot}5^\circ$, and continue to decrease with further lowering of temperature, until at temperatures below -6° extremely slow rates of change are observed.

SUMMARY.

1. During *post mortem* glycogenolysis in fish-liver at 0°, the following changes have been found to occur:

(*a*) An initial period of rapid loss of glycogen is followed by a period of decreased rate of loss, until the glycogen store becomes exhausted.

(*b*) No equally rapid formation of free sugar takes place. The concentration of sugar increases steadily at a lower rate and continues to increase despite total loss of glycogen. If sufficient time be allowed, a balance is eventually obtained between the glycogen loss and the gain in sugar *plus* lactic acid.

(*c*) The lactic acid concentration, although increasing steadily, remains at a comparatively low level throughout.

(*d*) No alteration in the total carbohydrate content is apparent until only a small concentration of glycogen remains in the tissue, after which point a steady decrease is observed.

These changes are independent of the presence of oxygen and indicate the rapid formation of an intermediary substance, which in its turn breaks down at a lower rate to form free sugar.

2. Investigation of the effect of freezing has shown that the conditions prevailing in liver tissue are different from those in muscle tissue, in so far as no increased rate of glycogenolysis occurs in the liver at freezing temperatures. On freezing at $-1{\cdot}5^\circ$ there is a marked decrease in glycogenolysis and further lowering of temperature causes a continually decreasing rate of change. At temperatures below -6° in the frozen tissue very small changes are evident over a period of 53 hours.

The author is indebted to the late Prof. J. J. R. Macleod for his interest and advice throughout the work.

REFERENCES.

Barrenscheen, Pany and Berger (1930). *Biochem. Z.* **229**, 196.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Holmes and Gerard (1929). *Biochem. J.* **23**, 738.
Sharp (1934). *Proc. Roy. Soc. Lond.* B **114**, 506.
Simpson and Macleod (1927). *J. Physiol.* **64**, 255.
Somogyi (1928). *J. Biol. Chem.* **78**, 117.
West, Scharles and Peterson (1929). *J. Biol. Chem.* **82**, 137.

CIII. THE ACTION OF CRYSTALLINE VITAMIN B_1 ON THE TISSUE OXIDATION OF CHRONIC AVITAMINOUS PIGEON'S BRAIN.

BY HÅKAN RYDIN.

From the Department of Biochemistry, Oxford.

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GAVRILESCU AND PETERS [1931, 1, 2] showed by Barcroft's method that the oxidation *in vitro* is reduced in avitaminous pigeon's brain tissue, suspended in glucose-phosphate solution as compared with normal controls. This particularly referred to the lower parts of the brain and not to cerebellum. The reduced oxygen uptake could be restored in part by the addition of a vitamin B_1 concentrate *in vitro*. On exchanging glucose for lactate [Gavrilescu *et al.*, 1932] these effects became more marked. No significant defect in the oxygen uptake and no vitamin effect were observed in brains from normal pigeons or from avitaminous ones in absence of substrate, or in the cases where this consisted of succinate. Passmore *et al.* [1933] showed a parallelism between crystalline vitamin B_1 and the concentrate previously used. The changes in oxygen uptake so produced by vitamin B_1 do not run parallel with oxidase estimations by reduction methods. By Thunberg's methylene blue technique, Rydin [1935] was not able to show any effect of crystalline vitamin B_1 on normal or on avitaminous tissues from brain, kidneys, liver and muscle, either in presence of the substrate (lactate or succinate) or in absence of these. In order to find out whether the results obtained by the Barcroft method are specific for the avitaminosis-B_1, or whether tissue from starved animals gives the same reactions, Gavrilescu and Peters [1931, 1] carried out tissue oxidation tests in glucose-phosphate solution on brains from pigeons which had been cured of the opisthotonus symptoms with vitamin concentrate. Even before an increase of the body weight began to set in, higher values of the oxygen uptake were obtained than in the case of avitaminous birds in head retraction. Later it was shown by Meiklejohn *et al.* [1932] with addition of vitamin B_1 and further control of water intake that lactate as substrate gave the same results. The increase of the tissue oxidation in cured pigeons was parallel with the disappearance of the nervous symptoms and independent of the nutritional condition. Peters and his co-workers conclude from their investigations that the reactions obtained on avitaminous pigeon's brain are specific to avitaminosis-B_1 and independent of the nutritional condition. Further support of this view was furnished by Sinclair [1933] who showed that vitamin B_1 increases the respiratory quotient *in vitro* on avitaminous pigeons in head retraction, but does not alter the R.Q. in tests on pigeons which are fed on polished rice and show a marked loss of weight but no polyneuritic symptoms. The same conclusions were arrived at by Abderhalden and Vlassopoulos [1931] by tissue oxidation experiments with Barcroft's method on brain, liver and kidneys though their experiments are really in disagreement with those previously quoted [Gavrilescu *et al.*, 1932]. Without addition of substrate they found lower oxygen uptake for tissues from avitaminous pigeons than from starved or normal birds. The differences for brain were not large, a decrease of −12 % for starving and −23 % for avitaminous birds as compared with the normal. [See also Galvao and Florence, 1934, and comments by Rydin.]

It has generally been concluded from the work quoted above and that of Kinnersley and Peters [1930] that there is a relation between the symptoms of head retraction and deficiency in the brain, especially the lower parts, of vitamin B_1. So far however only polyneuritic birds in head retraction have been used. It should be of interest to study the tissue oxidation in polyneuritic pigeons not showing head retraction but with other nervous symptoms such as leg weakness. These symptoms are present in pigeons which show a chronic avitaminous condition, generally when they have been fed on polished rice for a period of more than 30 days. I have therefore studied the action of crystalline vitamin B_1 on the tissue oxidation of brains from these birds, partly on the whole of the brain, partly on cerebrum and the optic lobes + "the rest" separately. The result was a significant vitamin B_1 effect in experiments with the whole of the brain and with cerebrum alone. The vitamin effect on the optic lobes + "the rest," on the other hand, was generally only slight. Also the vitamin effect during the first periods of reading up to ¾ hour was only little apparent but later on became more marked. The bearing of this work upon that of Meiklejohn, Passmore and Peters will be considered after describing the experiments.

EXPERIMENTAL.

The present investigations were carried out on brain tissue with Barcroft's method under the same general test conditions as apply to other experiments in this field made in this laboratory. These conditions have been changed somewhat during the progress of the work. It is now known that the presence of Na pyrophosphate [Peters and Sinclair, 1933] and "warm" mincing [Peters *et al.*, 1935] much improve the vitamin effect and that this is always more marked in the respiration period 1–2 hours. In order to obtain some basis for comparison of this work with that of Meiklejohn *et al.*, I have not used pyrophosphate and have adopted cool conditions for mincing, because their experiments were done in colder weather. For similar reasons the cerebellum has been excluded. The pigeons were fed on polished rice and were picked out for experiments after having been on this diet for at least 30 days, showing marked leg weakness but no trace of head retraction. The birds were killed by decapitation. The brain was quickly taken out, placed on an iced plate and finely minced with a blunt bone spatula, whereupon it was transferred to the bottles. The apparatus used for the determination of the oxygen uptake was of the Barcroft-Dixon type. Into each bottle were measured 3 ml. of a solution consisting of Ringer-phosphate (p_H 7·3) together with neutralised Na lactate. The latter was added in such quantity as to make the final concentration of the Ringer-phosphate solution 0·033*M*. The bottles were weighed before and after the addition of approximately 100 mg. of tissue. The roll of filter-paper was put in place and wetted with 2*N* KOH, and finally 2γ vitamin B_1 hydrochloride were added to every second bottle. (As regards the composition and preparation of the Ringer-phosphate solution as well as the preparation and administration of the vitamin solution see Passmore *et al.* [1933]). The bottles were then evacuated twice to 70 mm. Hg with subsequent refilling with oxygen. The apparatus was then transferred to the thermostat (temp. 38°) for determination of the tissue oxidation in the usual way. In the tables all the values are the mean of two determinations. The difference between these in no case exceeded 10 %. The oxygen consumption is expressed in µl. per g. tissue per hour. The first reading was made after 12 minutes, the second after 10 minutes and the subsequent readings at intervals of half an hour.

RESULTS.

Table I shows the action of vitamin B_1 on the tissue oxidation in experiments with the whole of the brain. In these experiments 8 pigeons were used. In all the readings the value of the figures was numerically higher for the oxygen uptake in presence of vitamin B_1, but in some cases the effect of the vitamin was doubtful or not at all observable during the first two periods of reading (¾ hour). The average increase of oxygen uptake due to vitamin B_1 for all the birds amounted to 17 % for the first period and to 16 % for the second period. During the third and the fourth periods the corresponding figures were 28 and 30 %. The vitamin effect set in as usual, the oxygen uptake owing to the vitamin did not decrease in proportion to the normal control but kept on a higher level during several periods. In many cases the tissue oxidation in presence of vitamin B_1 was decidedly higher than in the controls even during the first period. If the tissue oxidation in presence of vitamin is compared with that without vitamin during all the test periods, an average increase is obtained which amounts to 21 % for the addition of vitamin B_1 *in vitro* over the 1¾ hour period.

Table I. *Cerebrum and the lower parts mixed.*

O_2 uptake in µl./g./hr.

Exp.		Periods (hours)				Loss of weight	Symptoms
		0-¼	¼-¾	¾-1¼	1¼-1¾	%	No bird in head retraction, none could fly
1	L	3330	2450	1920	1510	38·5	Leg weakness not very apparent for the rest nothing remarkable
	L+V	3360	2820	2270	1850		
2	L	1180	830	650	590	42·5	Severe leg weakness. Bad walk. Rugged
	L+V	1340	900	690	650		
3	L	2200	1750	1300	1030	43·5	Moderate leg weakness and ataxic walk
	L+V	3020	2500	1910	1540		
4	L	2190	1570	1190	870	29·1	" "
	L+V	2640	2110	1810	1400		
5	L	2050	1800	1450	1160	48·9	Moderate leg weakness. Very ataxic walk. Rugged
	L+V	2330	2030	1700	1430		
6	L	1690	1780	1260	1090	55·1	Severe leg weakness. Cannot walk more than a few steps. Rugged
	L+V	1820	1870	1670	1260		
7	L	2450	1980	1450	1370	56·2	" "
	L+V	2980	2040	1820	1750		
8	L	2210	1740	1420	1040	29·7	Leg weakness not very apparent. Very ataxic walk
	L+V	2640	1920	1780	1350		
Av.	L	2160	1740	1330	1080	42·9	
	L+V	2520	2020	1700	1400		
Diff.	µl. O_2	360	280	370	320	—	
	%	16·7	16·1	27·8	29·6		

Table II illustrates the effect of vitamin B_1 on the oxygen uptake in different parts of the brain, and shows a comparison between the effect on cerebrum on the one hand, and on the optic lobes + the rest on the other. Fifteen pigeons were used in these experiments. For the cerebrum, the vitamin effect was approximately the same as that obtained for experiments on the whole brain.

The average vitamin action on the tissue oxidation during the first period was small but later on showed a great increase amounting to 35·5 % during the fourth period. The average vitamin effect for all the periods was 18 %. It varied however very considerably for the different pigeons. For the optic lobes + "the rest," the vitamin effect was considerably less. The highest average vitamin effect amounted to 11·7 % for period 3. The tests showed great individual variations. Thus pigeons 10 and 13 showed a large vitamin effect even on these parts of the brain. The increase of the tissue oxidation in presence of vitamin B_1 was statistically significant for the last two periods, both for the cerebrum and the lower parts.

Table II. *Cerebrum and the lower parts separately.*

O_2 uptake in μl./g./hr.

		Period (hours)								
		Cerebrum			Optic lobes + "the rest"					
Exp.		¼–¾	¾–1¼	1¼–1¾	¼–¾	¾–1¼	1¼–1¾	Loss of weight %	Temp. of the bird	Symptoms No bird in head retraction, none could fly
9	L	2010	1400	1130	1560	1380	910	46·6	39·1°	Moderate leg weakness. Moderately ataxic walk
	L+V	2300	1770	1690	1670	1310	1080			
10	L	1900	1800	1240	1020	620	600	12·3	39·6°	,, ,,
	L+V	2020	1570	1410	1400	990	810			
11	L	2000	1380	820	1460	1230	750	14·8	39·1°	,, ,,
	L+V	2280	1980	1330	1550	1400	900			
12	L	1960	1690	1050	1520	1320	790	29·3	38·9°	,, ,,
	L+V	2060	1890	1230	1560	1280	820			
13	L	2100	1600	1230	1400	1020	1060	40·0	39·4°	Moderate leg weakness. Very ataxic walk
	L+V	2160	1860	1550	1870	1540	1420			
14	L	1310	980	610	1370	980	680	51·3	38·6°	Severe leg weakness. Cannot walk more than a few steps. General condition bad. Rugged
	L+V	1760	1560	1070	1440	1160	810			
15	L	2560	2080	1440	1710	1480	1150	41·9	38·5°	Moderate leg weakness and moderately ataxic walk
	L+V	2730	2350	1950	1940	1610	1160			
16	L	1490	1320	1120	1180	900	820	47·6	39·9°	,, ,,
	L+V	1690	1310	1210	1210	1010	860			
17	L	2190	1550	1490	1340	1270	1120	46·4	38·8°	Moderate leg weakness. Very ataxic walk. Rugged
	L+V	2500	1980	1820	1610	1470	1230			
18	L	1830	1510	1120	1550	1260	1020	51·1	38·4°	,, ,,
	L+V	2390	2180	1850	1530	1300	990			
19	L	1450	1060	940	1100	900	780	31·7	39·3°	,, ,,
	L+V	1770	1390	1200	1080	930	830			
20	L	1350	1020	820	1000	870	600	39·3	39·1°	Severe leg weakness. Very ataxic walk
	L+V	1480	1240	980	1010	930	730			
21	L	1050	660	610	1140	710	640	34·0	38·4°	,, ,,
	L+V	1330	830	820	1130	800	690			
22	L	1780	1560	1040	1470	1030	830	48·7	38·2°	,, ,,
	L+V	2270	2030	1840	1510	1130	940			
23	L	2230	1690	1450	1890	1670	1320	26·2	39·9°	Leg weakness not very apparent. Rather good walk, not ataxic
	L+V	2450	1930	1770	2010	1700	1310			
Av.	L	1810	1420	1080	1380	1110	870	—	39·0°	
	L+V	2080	1730	1450	1500	1240	970			
Diff.	μl. O_2	270	310	370	120	130	100			
	%	14·9	21·8	34·3	8·3	11·7	11·5			

At first sight it may seem that Exps. 10, 13 and 17 contradict the hypothesis previously advanced in this laboratory that head retraction is associated with biochemical changes in the optic lobe + "the rest." If it were certain that these birds were showing no head retraction, then the hypothesis would have to be modified. However, in the case of the results for the optic lobe + "the rest" upon the pigeons with chronic leg weakness, 11–17 birds showed no larger vitamin effects during the first hour period than those which have been found for normals [Gavrilescu *et al.*, 1932]; differences between L and L + V are almost within the experimental error. It seems possible therefore that the exceptions were actually latent cases of head retraction. With weak birds, it is admittedly difficult to decide whether muscular weakness is not masking any opisthotonus. The preponderance of evidence in Table II is that the leg weakness is accompanied by negligible deficiency in vitamin B_1 in the optic lobes + "the rest," but by some demonstrable deficiency in the cerebrum. In my experiments during 1 hour period this may be reckoned as 17 %, whereas in the experiments of Meiklejohn *et al.* for cerebrum of pigeons with head retraction, which seem to be the most nearly comparable, it was 28 %. The cerebral deficiency seems therefore to be less for the chronic birds.

Discussion.

Previous work on the oxygen uptake of avitaminous pigeon's brain carried out for the purpose of clearing up the correlation between avitaminosis-B_1 and inanition, shows that these phenomena are independent of each other. The reduced tissue oxidation and the vitamin B_1 effect on avitaminous pigeon's brains seem to be directly correlated with the head retraction and independent of the nutritional condition. In my experiments on brains from avitaminous pigeons showing leg weakness but no head retraction, a clear vitamin effect was present in tests on the whole of the brain and on the cerebrum, but generally only a slight effect was observed on the lower parts (the optic lobes + "the rest"). The experiments consequently show that vitamin B_1 can increase the tissue oxidation *in vitro* even in brains from avitaminous pigeons, from which head retraction is absent but which have nervous symptoms in the form of leg weakness. So far as comparison can be made with tests in the laboratory carried out upon a different batch of pigeons at a different time, the vitamin B_1 deficiency is rather less for the cerebrum in "leg weakness" than in the "opisthotonus" cases examined. The "leg weakness" is not correlated with deficiency of vitamin B_1 in the optic lobes + lower parts, but there arises from my work the possible new point that the chronic symptoms may be partly central in origin. There is a high correlation between lowered vitamin B_1 in the cerebrum and these symptoms.

Summary.

1. The action of crystalline vitamin B_1 on the tissue oxidation of brains from chronic avitaminous pigeons showing leg weakness but no head retraction was studied by Barcroft's method in the presence of lactate and Ringer-phosphate solution.

2. Vitamin B_1 evidently increased the tissue oxidation in experiments on the whole of the brain and on cerebrum alone but generally only brought about a slight increase with the lower parts of the brain (the optic lobes + "the rest").

3. When compared with the results previously obtained in this laboratory from pigeons with head retraction, the vitamin effect was less marked. This particularly refers to the lower parts of the brain where the pigeons in head retraction showed a pronounced increase of the oxygen uptake with addition of vitamin *in vitro*.

I am very deeply indebted to Prof. Peters for his great interest and advice during the course of this work.

REFERENCES.

Abderhalden and Vlassopoulos (1931). *Pflüger's Arch.* **226**, 808.
Galvao and Florence (1934). *Pflüger's Arch.* **233**, 714.
Gavrilescu, Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **110**, 431.
—— and Peters (1931, 1). *Biochem. J.* **25**, 1397.
—— —— (1931, 2). *Biochem. J.* **25**, 2150.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 711.
Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **111**, 391.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Peters and Sinclair (1933). *Biochem. J.* **27**, 1677.
—— Rydin and Thompson (1935). *Biochem. J.* **29**, 53.
Rydin (1935). Unpublished results.
Sinclair (1933). *Biochem. J.* **27**, 1927.

CIV. THE POTENTIOMETRIC TITRATION OF SOLUTIONS OF VITAMIN B_1.

By RICHARD CAYLEY GIFFARD MOGGRIDGE
AND ALEXANDER GEORGE OGSTON.

From the Department of Biochemistry, Oxford.

(*Received February 28th, 1935.*)

THIS work forms part of a programme designed to throw light upon the constitution of vitamin B_1 prepared in this laboratory [Kinnersley *et al.*, 1935]; an unavoidable delay in starting gave us the advantage of having in our hands the results of a titration of Jansen's vitamin, privately communicated by Birch and Harris [Birch, 1934], when they wrote for a specimen of our vitamin to compare its behaviour with their previous results. We understand that they have this work in hand.

They obtained preliminary evidence of a dissociation constant at p_K 5·1 and another in the neighbourhood of p_K 9; that part of the titration curve representing the p_K 9 group was characterised by the fact that, on addition of acid or alkali, equilibrium was not immediately attained; a large change of p_H was produced immediately after each addition, but the p_H drifted back to more alkaline or acid values respectively. In the course of a complete titration from p_H 3 to 11 and back, about 20 % of the p_K 9 group was destroyed, the amount of the p_K 5·1 group remaining unchanged.

The properties of the p_K 9 group suggest at once those of a pseudo-acid converted to the acid form, or of a base converted to the pseudo-base form, as p_H is increased. It was desired if possible to determine the nature of this group. If this hypothesis is correct, the equilibrium may be represented, for the case of a pseudo-acid as

$$\mathrm{PA} \overset{K_1}{\rightleftarrows} \mathrm{HA} \overset{K_2}{\rightleftarrows} \mathrm{H^+} + \mathrm{A^-} \qquad \text{......(A).}$$

The overall dissociation constant K is given by

$$K = \frac{K_1 K_2}{1 + K_1} \qquad \text{......(B).}$$

It is apparent that any method for determining the nature of the dissociating group which relies on measuring the difference of K in two media, is not directly applicable here, since the effect of change of medium on K_1 is not measurable or predictable, so that the change of K_2 cannot be determined. It has however been found possible to determine K_2 independently of K_1 in water and 90 % alcohol.

EXPERIMENTAL.

The technique of these determinations is to be described elsewhere. The titration cells were constructed to hold a solution of 0·25 mg. in 2 ml. of solvent; the liquid junctions were made in sintered plugs of finely ground glass and were found to be reproducible to within 2 mv. Titrant solutions of strength $N/10$ were added from capillary micro-burettes. The p_H scales were standardised as described by Ogston and Brown [1935]; the glass membranes used with a Macfarlane-Pye valve potentiometer were standardised with buffer solutions checked against the hydrogen electrode. Stirring of the solutions used with the glass electrode was effected by bubbling nitrogen slowly through them. Blank experiments showed that the temperature of the room remained sufficiently constant for the accuracy required (18 ± 0·5°), and that any absorption of carbon dioxide from the air, even by alkaline solutions, was negligible.

The dissociation constants were calculated from the experimental values by the method described by Ogston and Brown [1935].

RESULTS.

Experiments were conducted both at 18° and at 0° using hydrogen and glass electrodes in aqueous solutions and glass electrodes in 90 % ethyl alcoholic solutions. The form of curve obtained by Birch and Harris was confirmed, with certain minor differences. The results of various experiments may be summarised as follows.

I. Using the hydrogen electrode, neutral solutions of the vitamin dihydrochloride in water at 18° were titrated with acid to p_H 3, then with alkali up to p_H 10 and again back to p_H 3 with acid.

(1) Between p_H 3 and 7 the curve can be described by two constants of p_K 2·5 and 4·7; on the other hand the initial curve obtained on first titrating to p_H 3 was never reproduced in subsequent titration of the same solution (Fig. 1).

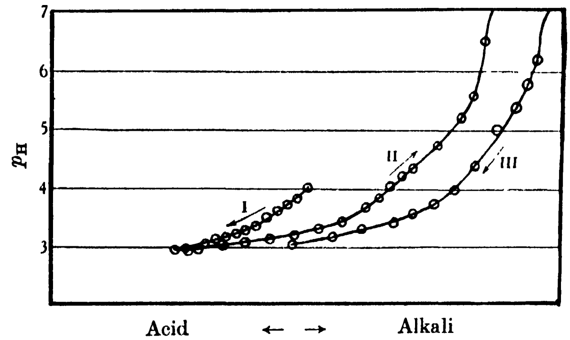


Fig. 1. Titration with hydrogen electrode at 18° (uncorrected). Curve I. Initial titration with acid. Curve II. Acid part of titration with alkali. Curve III. Return curve from p_H 10, with acid, showing shift to alkaline side.

(2) Between p_H 7 and p_H 10 the drifting of p_H after additions of titrant was confirmed; the time taken from each addition of titrant till equilibrium was established was about ¾ hour. Four such "equilibrium" curves gave values of p_K between 8·2 and 8·5, the mean being 8·3.

(3) Displacement of the curve as a whole to the alkaline side was observed on titrating back with acid, without change of its form, *i.e.* without alteration of the amount or dissociation constant of any of the groups titrated (Fig. 1). No disappearance of the p_K 8·3 group occurred.

II. Similar "equilibrium" titrations were carried out using glass electrodes in aqueous and in 90 % alcoholic solutions at 18°. The constants obtained in water were p_K 3·4, 4·8 and 9·1; in 90 % alcohol p_K 4·2 and 9·3 (values for the group having p_K 3·4 in water were not obtained since titration was not carried to a low enough p_H). The general behaviour was the same as with the hydrogen electrode with the following differences.

(1) The initial curve obtained by titrating with acid to p_H 3 (*cf.* I (1) above) was exactly reproduced on titrating back with alkali.

(2) The constants of two of the groups were different.

(3) The displacement of the curves as a whole (I (3) above) was not observed.

In 90 % alcoholic solutions the behaviour is similar to that in water except that in alkaline solution 2–3 hours are required for establishment of equilibrium after each addition of titrant.

III. Assuming the molecule of the vitamin to be $C_{12}H_{16}ON_4S.2HCl$, the amounts of the two basic groups were within 5 % of 1 equivalent per mol. The amount of the p_K 9 group was, using the hydrogen electrode, sometimes 1, sometimes 2 equivalents per mol.; using the glass electrode, always 2 equivalents per mol.

IV. Assuming the pseudo-acid or pseudo-base hypothesis to be correct, the slowness with which equilibrium is established should make it possible to titrate the vitamin in each form separately by performing rapid titrations. This was done using the glass and hydrogen electrodes; in order further to retard the attainment of equilibrium, the cell was immersed in melting ice (see Fig. 2 which illustrates the form of the curves obtained).

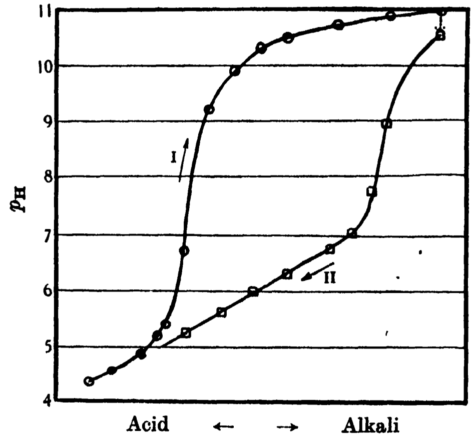


Fig. 2. Rapid titrations with the hydrogen electrode at 0° (uncorrected). Curve I. Rapid titration with alkali (identical with solvent blank). Curve II. Rapid titration with acid after establishment of equilibrium.

(1) Solutions of the vitamin dihydrochloride were titrated rapidly with alkali to p_H 11; the results were the same using either hydrogen or glass electrodes and showed the absence of any group having a p_K greater than 7 (*i.e.* the curves were identical with the water blank).

(2) Solutions of the vitamin were brought rapidly to p_H 11 with alkali and allowed to stand until equilibrium was established (as before, the p_H fell steadily during this process); they were then rapidly titrated back with acid. The resulting curves gave constants p_K 6·8 in water, and p_K 7·9 in 90 % alcohol. With the hydrogen electrode a p_K of 6·4 was obtained (*cf.* II (2)).

V. The colour reaction test was performed on the residual solutions from all runs; where the hydrogen electrode had been used the reaction showed only 5–10 % of the strength expected from the amount of vitamin in solution; where glass electrodes had been used, the colour reaction showed about 30 % of the expected value.

The constants obtained are summarised in the table.

Table I.

	p_K			$\Delta\ p_K$ (glass) H_2O - 90 % EtOH
	H_2.Pt in H_2O	Glass in H_2O	Glass in 90 % EtOH	
Basic group 1. 18°	2·5	3·4	—	—
Basic group 2. 18°	4·7	4·8	4·2	-0·6
Acid group. Equilibrium p_K 18°	8·3	9·1	9·3	+0·2
p_{K_2} 0°	6·4	6·8	7·9	+1·1

DISCUSSION.

There is a marked difference of behaviour of the vitamin solutions with hydrogen and glass electrodes; with glass, their behaviour is more reproducible, but apart from this the main differences are:

(1) The difference between p_K 2·5 and p_K 3·4 for the weaker basic group, together with the anomaly of the initial part of the curve (I (1) and Curve I); both these can be accounted for by conversion of this group by action of the hydrogen electrode into one having a lower p_K.

(2) The difference found in the colour reaction (V).

(3) The displacement of the whole curve towards the alkaline side (I (3)).

(4) The difference between p_K 8·3 and 9·1 for the constant in this region.

(5) The difference between the amounts of the p_K 8–9 group, using hydrogen and glass electrodes (IV).

Some of these anomalies are unexplained, but in view of the present scarcity of material it was thought that further work was not justified. However, it seems likely that the vitamin is reduced by the hydrogen electrode, so that the results from the glass electrode best represent its behaviour: the group concerned in the colour reaction can be none of those titrated.

It was found that the vitamin does not poison the hydrogen electrode, nor does it appear to be strongly adsorbed.

The two acid-binding groups of the vitamin have p_K values of 3·4 and 4·8 respectively; this is in accord with the chemical data on the formation of hydrochlorides and sulphates [Kinnersley *et al.*, 1935]. The change of the second from p_K 4·8 to 4·2 between water and 90 % alcohol is of the order to be expected of a basic nitrogen atom [Neuberger, 1934]. Levene and Sims [1925] and Levene *et al.* [1926] have shown that pyrimidine amino-groups have p_K values of about this amount (4·0–4·6), so that the group of p_K 4·8 might be identified with the pyrimidine amino-group of Williams's [1935] formula. However, the quaternary nitrogen demanded by his formula is in disagreement with the evidence, since this must be a third, strongly basic group which together with the groups of p_K 3·4 and 4·8 would cause the vitamin to combine with three equivalents of acid instead of the two equivalents observed.

It appears that the vitamin has also a pseudo-acid group, in view of the following facts.

(1) Apart from the two basic groups (p_K 3·4 and 4·8) the form of the vitamin stable in acid solution contains no acid or basic groups titrating between p_H 7 and 11 (IV (1) above).

(2) Alkali causes the reversible formation of a group having p_K 6·8 in water and 7·9 in 90 % alcohol (IV (2) above). This change of p_K is characteristic of an enolic acid (unpublished observations of one of the authors).

This might be ascribed to a keto-enol change but for the fact that there appear two equivalents of acid per mol. of vitamin. The splitting of an oxide ring or of a sulphide ring (as in Williams's formula) might account for the facts, provided that the dissociation constants of the two —OH groups, or of the —OH and —SH so formed, were not very different. The possibility of hydrolysis of a —CO—NH— linkage seems to be excluded. The formation of a pseudo-base from quaternary nitrogen, such as that found in nicotine derivatives by Lowry and Lloyd [1932], will not account for the present results, both in view of the amount of the group (two equivalents per mol.), and in view of the change of p_K found between water and 90 % alcohol.

The titration results can be correlated with the absorption spectra of the vitamin [Peters and Philpot, 1933; Holiday, 1935] on the assumption that the labile band observed on bringing the vitamin from acid solution to p_H 9·5 is due to the unionised pseudo-acid form; as conversion of PA (equation A) to HA and A^- occurs, this band disappears.

SUMMARY.

Potentiometric titrations have been performed on solutions of crystalline vitamin B_1 dihydrochloride. Evidence has been obtained of the existence of two weakly basic groups of p_K 3·4 and 4·8, and of a pseudo-acid group titrating in the region of p_H 9. The dissociation constant of the acid form of the vitamin has the value p_K 6·8 in water at 0°.

The authors wish to express their thanks to Mr Kinnersley and to Mr Philpot for help and advice; and to Prof. Peters, at whose suggestion this work was undertaken, for invaluable advice throughout its course and for measurement of the colour reactions.

REFERENCES.

Birch (1934). Thesis, Cambridge.
Holiday (1935). *Biochem. J.* **29**, 718.
Kinnersley, O'Brien and Peters (1935). *Biochem. J.* **29**, 716.
Levene, Bass and Sims (1926). *J. Biol. Chem.* **70**, 229.
—— and Sims (1925). *J. Biol. Chem.* **65**, 522.
Lowry and Lloyd (1932). *J. Chem. Soc.* 1626.
Neuberger (1934). *Proc. Roy. Soc. Lond.* B **115**, 180.
Ogston and Brown (1935). *Trans. Faraday Soc.* In the press.
Peters and Philpot (1933). *Proc. Roy. Soc. Lond.* B **113**, 48.
Williams (1935). *J. Amer. Chem. Soc.* **57**, 229.

CV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

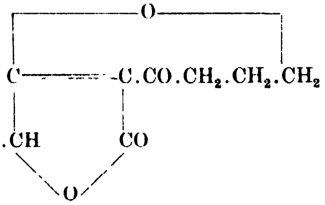
XLIII. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH. III. THE MOLECULAR CONSTITUTION OF CARLIC AND CARLOSIC ACIDS.

By PERCIVAL WALTER CLUTTERBUCK, HAROLD RAISTRICK AND FRITZ REUTER.

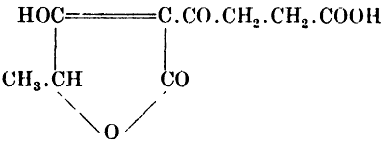
From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received February 22nd, 1935.*)

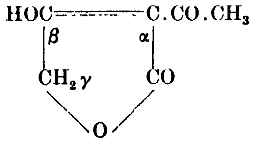
It was recently shown [Clutterbuck *et al.*, 1934] that *Penicillium Charlesii* G. Smith, a new species of *Penicillium* isolated by our late colleague, Mr J. H. V. Charles, from mouldy Italian maize, produced from glucose a number of hitherto undescribed organic acids. Of the latter, carolic acid, $C_9H_{10}O_4$, and carolinic acid, $C_9H_{10}O_6$, which were obtained in the largest yields, have been shown to have the constitutions I and II respectively [Clutterbuck *et al.*, 1935]:



I. Carolic acid.



II. Carolinic acid.



III. α-Acetyltetronic acid.

The acids may be regarded, therefore, as condensation products of *l*-γ-methyltetronic acid with γ-hydroxybutyric (butyrolactone) and succinic acids respectively, condensation with elimination of water occurring between a carboxyl group of the latter acids and an α-hydrogen atom of the γ-methyltetronic acid ring.

Both acids showed somewhat unusual properties. They were broken down quantitatively with great ease on boiling with dilute mineral acids giving 1 mol. each of acetoin and CO_2, carolic acid giving also 1 mol. of butyrolactone, and carolinic acid 1 mol. of succinic acid. Both acids on bromination gave *d*-α-bromo-γ-methyltetronic acid, the α-side-chains being removed. Both were readily reduced with Pd and hydrogen, the CO group of the α-side-chain being converted into a CH_2 group, and both gave with diazomethane methyl ethers which were attacked by NaOH with remarkable ease. They also showed rather characteristic behaviours with 2:4-dinitrophenylhydrazine. Thus carolic acid gave no visible immediate reaction with this reagent in $2N$ HCl but slowly over a period of 8 weeks a precipitate separated which at first consisted of a mixture of mono- and bis-dinitrophenylhydrazones of $C_9H_{10}O_4 + H_2O$ and later became the pure bis-derivative. The monodinitrophenylhydrazone was soluble in $NaHCO_3$ and fairly soluble in water, the bis-derivative being insoluble in either reagent. Carolinic acid immediately gave a yellow monodinitrophenylhydrazone, insoluble in water but soluble in $NaHCO_3$, which was not converted into the bis-derivative on standing with excess of reagent.

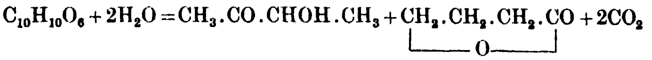
It was found that α-acetyltetronic acid (III), which also contains the α-keto-substituted-tetronic acid nucleus, reacted in a manner showing an absolute analogy with the behaviour of carolic and carolinic acids.

In our first work [Clutterbuck *et al.*, 1934], in addition to carolic and carolinic acids, two further acids, each containing ten carbon atoms, *viz.* carlic acid, $C_{10}H_{10}O_6$, and carlosic acid, $C_{10}H_{12}O_6$, were also obtained in smaller yields. These acids have now been shown to be closely related in constitution to carolic and carolinic acids, and it is the purpose of this paper to describe the investigation of the molecular constitution of carlic and carlosic acids.

Carlic acid, $C_{10}H_{10}O_6$, M.P. 176°, $[\alpha]_{5461} - 160°$ (in water), gives with $FeCl_3$ an immediate brownish yellow precipitate soluble in excess to an orange-yellow solution. It does not give a colour with $NaNO_2$. The acid blues Congo red and titrates in water as a dibasic acid. It does not however contain any active hydrogen atom, as determined in dry anisole by the Zerewitinoff method (Roth), whilst it contains two active hydrogen atoms when determined in pyridine. It appears therefore that carlic acid, like carolic acid, must contain a ring system which opens up in aqueous solution, whilst enolisation must occur in pyridine. A number of unusual enolisations of the CO groups of acid anhydrides (containing a $CH_2.CO$ group) in pyridine with activation of hydrogen have been recently reported [Ishikawa and Kojima, 1934].

Carlosic acid, $C_{10}H_{12}O_6$, M.P. 181°, $[\alpha]_{5461} - 160°$ (in water) gives with $FeCl_3$ an immediate brownish yellow precipitate insoluble in excess. It does not give a colour with $NaNO_2$. The acid blues Congo red and titrates in water as a dibasic acid. It gives an amount of methane corresponding to 2·2 (at 18°) to 2·5 (at 95°) active hydrogen atoms in dry anisole and 2·1 (at 18°) and 2·9 (at 95°) in pyridine as determined by the Zerewitinoff method (Roth).

On hydrolysis by boiling with $2N$ H_2SO_4 carlic and carlosic acids, like carolic and carolinic acids, very quickly give 1 mol. of CO_2. Carlic and carlosic acids differ however from carolic and carolinic acids in that on continued hydrolysis they both slowly give a second molecule of CO_2 over a period of about 25 hours. Under these conditions of protracted hydrolysis, carlic acid gives 1 mol. each of acetoin and butyrolactone with 2 mols. of CO_2.

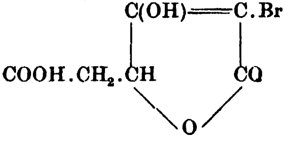


$$C_{10}H_{10}O_6 + 2H_2O = CH_3.CO.CHOH.CH_3 + \underbrace{CH_2.CH_2.CH_2.CO}_{O} + 2CO_2$$

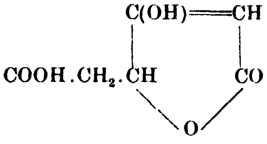
whilst carlosic acid gives 1 mol. each of acetoin and n-butyric acid with 2 mols. of CO_2.

$$C_{10}H_{12}O_6 + 2H_2O = CH_3.CO.CHOH.CH_3 + CH_3.CH_2.CH_2.COOH + 2CO_2.$$

Carlic and carlosic acids on bromination in dilute acetic acid give the same product, $C_6H_5O_5Br$, M.P. 194°, which has been shown to be very probably l-α-bromo-γ-carboxymethyltetronic acid (IV) and which on catalytic reduction with palladium and hydrogen is readily debrominated giving *l*-γ-carboxymethyltetronic acid (V).



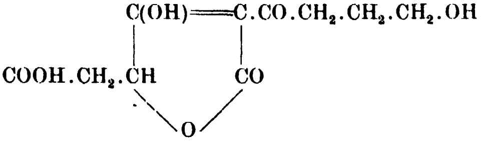
IV.



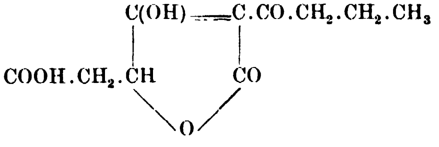
V.

This acid, on hydrolysis with 2*N* H_2SO_4, loses 1 mol. of CO_2 very readily as do carolic, carolinic and γ-methyltetronic acids, whilst a second molecule of CO_2 is lost more slowly, the behaviour being exactly parallel with that of carlic and carlosic acids. Protracted hydrolysis also results in the formation of 1 mol. of acetoin. It is therefore reasonable to suppose that the acetoin and 2 mols. of CO_2, obtained on hydrolysis of carlic and carlosic acids, arise from the *l*-γ-carboxymethyltetronic acid nuclei present in these acids and separated from them on bromination.

Carlic and carlosic acids therefore result from the condensation of 1 mol. of *l*-γ-carboxymethyltetronic acid with 1 mol. of γ-hydroxybutyric acid (butyrolactone) and 1 mol. of *n*-butyric acid respectively. Since synthetic α-acetyltetronic acid has been shown on bromination to lose the α-side-chain giving α-bromotetronic acid, and since carolic, carolinic, carlic and carlosic acids all lose their side-chains on bromination giving α-bromotetronic acid derivatives, it is reasonable to suppose that in the four metabolic acids the side-chains are attached to the α-carbon of the substituted tetronic acid ring as in α-acetyltetronic acid. This view is further supported by the complete analogy in properties between the four metabolic acids and α-acetyltetronic acid, and by their absorption spectra which have been investigated by Dr E. L. Hirst of Birmingham University, and will be reported in a subsequent publication. The following formulae have therefore been adopted for carlic acid (+H_2O) (VI) and carlosic acid (VII) respectively.

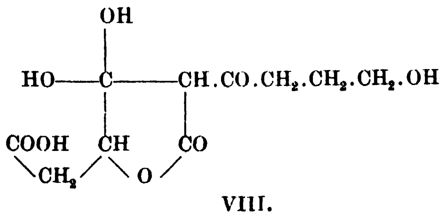


VI. Carlic acid (+H_2O).

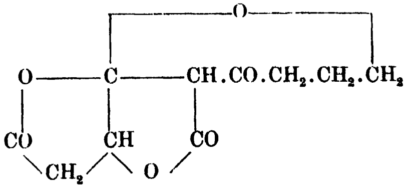


VII. Carlosic acid.

The structural formula of carlic acid then requires the removal of 1 mol. of H_2O from VI. Carlic acid titrates as a dibasic acid in water but contains almost no active hydrogen (in anisole) and two active hydrogen atoms in pyridine. The most probable formula for carlic acid, in view of these facts, is then given by IX, which is derived by hydration of the β-keto group of the tetronic acid ring (VIII) followed by double ring closure.



VIII.



IX. Carlic acid.

This structure would give practically no active hydrogen in anisole, but by analogy with the compounds investigated by Ishikawa and Kojima [1934] would show two active hydrogen atoms in pyridine due to enolisation of the two CO groups having CH_2 groups adjacent to them.

It now remains to be shown that these constitutional formulae are in full agreement with all the known properties of these acids.

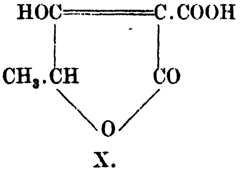
(*a*) *General properties of carlic and carlosic acids.* The above formulae for carlic and carlosic acids would obviously permit optical activity, and in view of the stable substitution of the α-carbon of the tetronic acid ring, we should not expect a colour with $NaNO_2$. The fact that both acids can be recovered unchanged after boiling with acetyl chloride is explained by the fact that all the hydroxyl groups are of the keto-enol type and revert to the ketonic form in presence of this reagent. With carlic acid we should not expect any active hydrogen in dry anisole, but there is the possibility of two enolic groups being formed in pyridine, thus accounting for the two active hydrogen atoms. With carlosic acid, two active hydrogens would be expected with certainty and a third might arise by enolisation in the side chain. The value found in anisole was 2·5. Both acids however on titration with aqueous sodium hydroxide would react as dibasic acids, if it be assumed that with carlic acid two rings open at the β-carbon of the tetronic acid ring.

(*b*) *Bromination of carlic and carlosic acids.* On bromination in approximately 50 % acetic acid under the conditions used for carolic and carolinic acids, carlic and carlosic acids gave the same bromo-derivative, $C_6H_5O_5Br$. This compound gave a red colour with $FeCl_3$ and a violet colour with $NaNO_2$, the latter reaction being characteristic of tetronic acids having the α-CH_2 either unsubstituted or substituted by an easily removable substituent, *e.g.* bromine. It titrated as a dibasic acid and on reduction with palladium-charcoal-hydrogen it readily gave the corresponding dibasic acid $C_6H_6O_5$. This acid also gave the same $FeCl_3$ and $NaNO_2$ colour reactions as did the brominated acid, and on hydrolysis with 2*N* H_2SO_4 very quickly gave 1 mol. of CO_2 and then much more slowly a second molecule of CO_2, whilst after complete hydrolysis, 1 mol. of acetoin remained. The hydrolysis therefore follows the equation

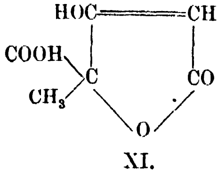
$$C_6H_6O_5 + H_2O = CH_3.CHOH.CO.CH_3 + 2CO_2.$$

The acid $C_6H_6O_5$ differs from γ-methyltetronic acid, $C_5H_6O_3$, by CO_2 and by titrating as a dibasic acid, whereas the latter acid is monobasic. The absorption spectrum of the acid $C_6H_6O_5$, as will be shown in the later publication previously referred to is almost identical with that of γ-methyltetronic acid.

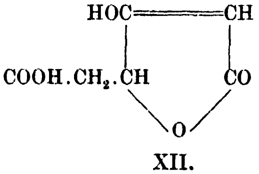
It seems reasonable to suppose therefore that the acid $C_6H_6O_5$ is a carboxy-derivative of γ-methyltetronic acid. Three formulae are conceivable, X, XI, XII.



X.

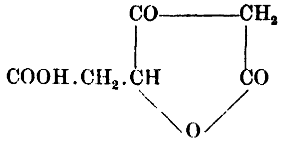


XI.



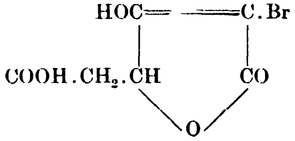
XII.

Formula X is however excluded since this acid was an intermediate substance in the synthesis of γ-methyltetronic acid and was shown to exist only as the ester or as salts, CO_2 being lost immediately on acidification [Benary, 1911; Clutterbuck *et al.*, 1935]. Our acid however is quite stable in acid solution at room temperature and is stable to heat up to its M.P. of 182°. Formula XI appears to be impossible also for a similar reason, since opening the tetronic acid ring would lead to a β-ketodicarboxylic acid. We have seen however that on hydrolysis the acid loses 1 mol. of CO_2 very quickly in the same way as γ-methyltetronic acid, whilst the second molecule of CO_2 is only lost slowly. This is best explained in terms of formula XII when the reaction becomes:



$$\begin{aligned}+\mathrm{H_2O} &= \mathrm{COOH.CH_2.CHOH.CO.CH_2.COOH}\\ &= \mathrm{COOH.CH_2.CHOH.CO.CH_3 + CO_2}\\ &= \mathrm{CO_2 + CH_3.CHOH.CO.CH_3 + CO_2.}\end{aligned}$$

Here 1 mol. of CO_2 is lost at the same rate as with γ-methyltetronic acid in each case from the COOH group in the β-position to the CO group, whilst the second molecule of CO_2 is lost more slowly from the COOH group in the γ-position to the CO group in the acid $C_5H_8O_4$. Compounds with formulae X and XI also would hardly be expected to be stable to heat up to a M.P. of 182°. The acid $C_6H_6O_5$ is therefore regarded as γ-carboxymethyltetronic acid XII, and its bromo-derivative will therefore have the structure XIII.



XIII.

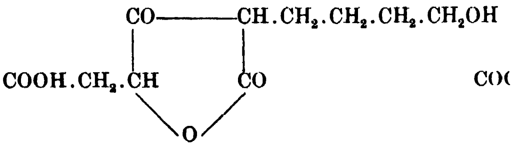
It appears therefore that during bromination of carlic and carlosic acids in dilute acetic acid the α-side-chains are removed and replaced with bromine in a way entirely analogous to that with synthetic α-acetyltetronic acid and with carolic and carolinic acids.

(*c*) *Hydrolysis of carlic and carlosic acids.* The ready hydrolysis of these acids with boiling 2*N* H_2SO_4 has already been discussed and brings them into line with carolic, carolinic and α-acetyltetronic acids. This property is undoubtedly due to the α-ketotetronic acid structure which is common to all five acids, and which on opening the tetronic acid ring represents them as derivatives of diacetylacetic acid.

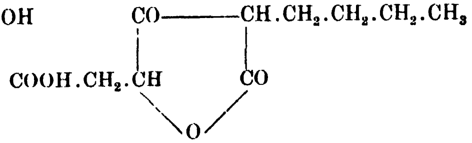
(*d*) *Reduction of carlic and carlosic acids.* Carlic acid, $C_{10}H_{10}O_6$, on reduction with palladium-charcoal-hydrogen, absorbs $2H_2$ and gives the reduced dibasic acid $C_{10}H_{14}O_6$. This result is explained, as with carolic acid, by assuming that on dissolving in water 1 mol. of H_2O is added, and that the CO group of the side-chain is reduced to a CH_2 group.

Carlosic acid, $C_{10}H_{12}O_6$, on reduction absorbs $2H_2$, giving the reduced dibasic acid $C_{10}H_{14}O_5$, and behaves exactly as do carolinic and α-acetyltetronic acids. Here again therefore the reaction is merely the reduction of the CO group of the side-chain to a CH_2 group.

Reduced carlic and carlosic acids may therefore be represented by structures XIV and XV.



XIV.



XV.

Like reduced carolic, carolinic and α-acetyltetronic acids, reduced carlic and carlosic acids give red $FeCl_3$ colours identical with the colour given by γ-methyltetronic, α-ethyltetronic and acetoacetic acids. The reduced acids, as also reduced carolic and carolinic acids, hydrolyse very slowly with boiling $2N$ H_2SO_4 and give substances which reduce Fehling's solution in the cold, slowly give bis-dinitrophenylhydrazones, and probably therefore contain the CHOH.CO grouping. This confirms the view that the tetronic acid ring itself is not reduced under our conditions and moreover, γ-methyltetronic acid itself does not reduce with our catalyst.

(*e*) *Absorption spectra of carlic and carlosic acids and of the corresponding reduced acids.* As will be shown in the subsequent publication previously referred to, the structures assigned to these acids fit the requirements of their absorption spectra. Thus carlic and carlosic acids, like carolic, carolinic and α-acetyltetronic acids, all give a double-banded spectrum in aqueous solution. On the other hand, reduced carlic and carlosic acids, like reduced carolic and carolinic acids, give a single band corresponding to that of either γ-methyl- or α-ethyl-tetronic acid. Thus reduction has resulted in the loss of the ketonic absorption band.

(*f*) *Reactions of carlic and carlosic acids with 2:4-dinitrophenylhydrazine.* In our earlier paper it was shown that α-acetyltetronic and carolinic acids immediately gave yellow monodinitrophenylhydrazones. Carolic acid, on the other hand, gave no immediate precipitate, probably owing to the solubility of the monodinitrophenylhydrazone. After 48 hours a precipitate slowly began to separate which at first consisted of a mixture of mono- and bis-derivatives and later became pure bis-dinitrophenylhydrazone.

Carlic acid gave no precipitate even after 2 weeks and only after 8 weeks showed a small separation of crystals which however proved to be the bis-dinitrophenylhydrazone of diacetyl. Carlic acid must therefore very slowly have been degraded by acid hydrolysis. A similar behaviour has been observed with γ-carboxymethyltetronic acid.

Carlosic acid, like carolinic and α-acetyltetronic acids, gave an immediate precipitate of the monodinitrophenylhydrazone. This derivative was also the only substance isolated after long standing with the reagent. Small amounts of other substances were formed, probably by degradation of carlosic acid, but were not characterised.

(*g*) *Reactions of carlic and carlosic acids with diazomethane.* Carlic acid, like carolic acid, on treatment with diazomethane in incompletely dry ether, very slowly reacted and, after standing overnight, gave a yellow oil which had a methoxyl content rather greater than that of a dimethyl derivative. The elements of water must therefore have been added, and a methyl ether methyl ester have been formed.

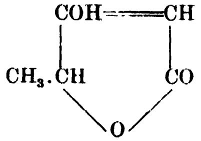
Carlosic acid under the same conditions reacted immediately, and gave an oil which had a methoxyl content also high for that of a dimethyl compound, presumably a methyl ether methyl ester.

As with the methyl derivatives of α-acetyltetronic, carolic and carolinic acids, both these oils were readily hydrolysed by N NaOH at the ordinary temperature, the methyl ether grouping again being attacked.

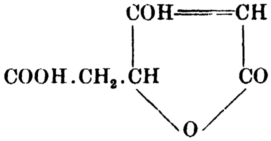
The fact that all these acids gave methyl derivatives having high methoxyl values suggests that the α-CO group of the side-chain undergoes enolisation and subsequent methylation to a slight extent.

This close similarity in behaviour between carlic and carlosic acids on the one hand and α-acetyltetronic, carolic and carolinic acids on the other hand, supported by the absorption spectra data makes it very probable that in carlic ($+H_2O$) and carlosic acids, γ-hydroxybutyric and *n*-butyric acids respectively are condensed with *l*-γ-carboxymethyltetronic acid to give the ketonic structures VI and VII similar to α-acetyltetronic acid. These formulae have therefore been allotted to carlic ($+H_2O$) and carlosic acids respectively, which may therefore be formulated as α-[γ-hydroxybutyryl]-γ-carboxymethyltetronic acid and α-butyryl-γ-carboxymethyltetronic acid.

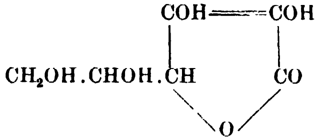
A further point of interest is the close relationship of these products to ascorbic acid (vitamin C). Thus carolic and carolinic acids both contain the γ-methyltetronic acid nucleus (XVI), carlic and carlosic acids the γ-carboxymethyltetronic acid nucleus (XVII), both of which show close analogy with the structure of ascorbic acid (XVIII).



XVI.



XVII.



XVIII.

All these acids have similar absorption spectra and show similar shifts of the absorption band in acid and alkaline solution.

EXPERIMENTAL.

Carlic acid, $C_{10}H_{10}O_6$, M.P. 176°, $[\alpha]_{5461}-160°$ (in water) gives with $FeCl_3$ an immediate brownish yellow precipitate soluble in excess to an orange-yellow solution. It does not give a colour with $NaNO_2$ but blues Congo red and titrates sharply in water as a dibasic acid. Thus 0·1214 g. required 10·70 ml. *N*/10 NaOH corresponding to an equivalent of 113·4. ($C_{10}H_{10}O_6$ titrating as a dibasic acid requires 113.) It does not contain any active hydrogen as determined in dry anisole by the Zerewitinoff method (Roth), 6·78 mg. at 28° giving only 0·04 ml. CH_4. In dry pyridine however it contains two active hydrogen atoms, 8·955 mg. at 28° giving 1·71 ml. CH_4, corresponding to 1·93 active hydrogen atoms. Carlic acid, like carolic acid, must therefore have a ring system which opens on dissolving in water and titrates, and in pyridine two carbonyl groups must enolise.

In regard to the yield of carlic acid, it was noted in our first paper that the acidified metabolism solutions after extraction 10–12 times with an equal volume of ether, and subsequently 5–6 times with chloroform, still had a negative rotation, and it was suggested that this was probably due to residual carlic acid. It has now been found that by adding still larger amounts of HCl for acidification and extracting with chloroform up to 30 times that the yield of carlic acid can be more than doubled.

Carlosic acid, $C_{10}H_{12}O_6$, M.P. 181°, $[\alpha]_{5461}-160°$ (in water) gives with $FeCl_3$ an immediate brownish yellow precipitate insoluble in excess. It does not give a colour with $NaNO_2$. It blues Congo red and titrates as a dibasic acid. Thus 0·1040 g. required 9·20 ml. *N*/10 NaOH corresponding to an equivalent of 113·1. ($C_{10}H_{12}O_6$ titrating as a dibasic acid requires 114.) In a determination of active hydrogen in dry anisole by the Zerewitinoff method (Roth) 8·46 mg. gave 1·86 ml. CH_4 at 18° and 2·10 ml. CH_4 at 95°, corresponding to 2·2 and 2·5 active hydrogen atoms respectively, while in pyridine 7·515 mg. gave 1·55 ml. CH_4 at 18° and 2·12 ml. CH_4 at 95° corresponding to 2·1 and 2·9 active hydrogen atoms respectively.

Both of these acids fail to give benzoyl-, *p*-nitro- or *p*-bromo-benzoyl-derivatives and can be recovered unchanged after boiling for 1 hour with acetyl chloride. They probably do not contain therefore under these conditions a free hydroxyl group.

It has been confirmed that carlosic acid, which is the most highly reduced of these acids, is only obtained when Czapek-Dox medium is used and not with Raulin-Thom solution. It is interesting to note that the metabolism on the latter medium proceeds twice as quickly as on the former medium.

A. *Bromination of carlic and carlosic acids. Preparation of* l-*α-bromo-γ-carboxymethyltetronic acid.*

1 g. of carlic or carlosic acid was dissolved in 15 ml. 50 % aqueous acetic acid and a standardised solution of bromine in the same solvent added. Immediate decoloration of the bromine occurred and a sharp end-point was obtained after the equivalent of 2 mols. of bromine had been added. The solution was then rapidly evaporated to dryness over sticks of KOH in a high vacuum at room temperature. A crystalline mass remained, weighing in each case 1 g. After recrystallisation from boiling ether-light petroleum the product in both cases melted at 194° (decomp.) and on mixing the two samples there was no depression of M.P. The acid gave a red $FeCl_3$ colour and a violet $NaNO_2$ colour. (Found (Schoeller): C, 30·44; H, 2·23; Br, 33·61 %. $C_6H_5O_5Br$ requires C, 30·39; H, 2·13; Br, 33·72 %.)

0·1045 g. on neutralisation in aqueous solution to phenolphthalein required 8·80 ml. *N*/10 NaOH corresponding to an equivalent of 119 ($C_6H_5O_5Br$ titrating as a dibasic acid requires 118·5). Rotation in water ($c = 0{\cdot}562$) $[\alpha]_{5461} - 117°$.

Reduction of l-*α-bromo-γ-carboxymethyltetronic acid, $C_6H_5O_5Br$, to an acid $C_6H_6O_5$ shown to be* l-*γ-carboxymethyltetronic acid.* *l*-α-Bromo-γ-carboxymethyltetronic acid, 0·25 g., was reduced catalytically using palladium-charcoal-hydrogen. The reduction, which was carried out as with α-bromo-γ-methyltetronic acid was complete in about 5 minutes and corresponded to an uptake of 1 mol. of hydrogen per mol. of substance reduced. The catalyst was filtered off and washed with 2*N* NaOH and then with water. The filtrate and washings were combined, acidified and extracted five times with an equal volume of ether, the ethereal extracts dried over anhydrous $MgSO_4$ and the ether removed *in vacuo*. A crystalline product remained which after recrystallisation from boiling ether-light petroleum melted at 182°. The acid gave a red colour with $FeCl_3$ and a violet one with $NaNO_2$. (Found (Schoeller): C, 45·61; H, 3·90 %. $C_6H_6O_5$ requires C, 45·55; H, 3·83 %.) 0·0129 g. on neutralisation in aqueous solution to phenolphthalein required 1·60 ml. *N*/10 NaOH corresponding to an equivalent of 80·6 ($C_6H_6O_5$ titrating as a dibasic acid requires 79). Rotation in water ($c = 0{\cdot}483$) $[\alpha]_{5461} - 52°$.

l-γ-Carboxymethyltetronic acid does not give an immediate precipitate with Brady's reagent, but during 6–8 weeks a precipitate slowly separated which proved however to be the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°, not depressed by mixing with an authentic specimen. Complete hydrolytic decarboxylation had therefore occurred.

Hydrolysis of l-*γ-carboxymethyltetronic acid, $C_6H_6O_5$.* 0·205 g. of the acid was heated with 20 ml. 2*N* H_2SO_4 in a stream of CO_2-free nitrogen exactly as in the hydrolysis of carolic acid, the resultant gases being passed through a dinitrophenylhydrazine bubbler and then through baryta bubblers. The amounts of CO_2 collected in 3, 9 and 17 hours respectively corresponded to 27·8, 40·8 and 51·8 ml. *N*/10 baryta. The theoretical amount of CO_2 assuming that 1 mol. of

$C_6H_6O_5$ gives 1 mol. of CO_2 requires 25·95 ml. $N/10$ acid. It is obvious therefore that 1 mol. of CO_2 is very readily liberated, the time taken corresponding exactly with those for carolic, carolinic, γ-methyltetronic and α-acetyltetronic acids, whilst a second molecule of CO_2 is liberated much more slowly. The hydrolysate was cooled and after neutralisation with concentrated KOH made up to 100 ml.; aliquot portions were used for the following tests. The solution gave a positive Voges-Proskauer reaction, reduced Fehling's solution in the cold, slowly in the manner characteristic of acetoin gave the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (not depressed by mixing with an authentic specimen), and on determination of the acetoin content by the Wood-Ost method was shown to contain 0·098 g. acetoin (theory for 1 mol. acetoin, 0·1142 g.).

As happens in all the hydrolysis experiments in which acetoin is formed, a little of the acetoin passed through the condenser in the stream of nitrogen, and the dinitrophenylhydrazine bubbler on standing gave slowly a very small amount of precipitate which proved to be the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°. If, however, the hydrolysis product had been diacetyl and not acetoin a very copious precipitate would have been formed in the dinitrophenylhydrazine bubbler, as control experiments have proved.

The acid $C_6H_6O_5$ behaves therefore like γ-methyltetronic acid in giving a red colour with $FeCl_3$, a violet one with $NaNO_2$ and on hydrolysis giving readily 1 mol. of acetoin and 1 mol. of CO_2, but differing in titrating as a dibasic (instead of monobasic) acid and in slowly giving on hydrolysis a second molecule of CO_2. As shown in the theoretical part of this paper, these facts fit best the view that the structure of this acid is γ-carboxymethyltetronic acid (XII), whilst the bromide is α-bromo-γ-carboxymethyltetronic acid (XIII).

B. *Hydrolysis of carlic acid.*

0·8114 g. carlic acid, M.P. 176°, was hydrolysed as with carolic and carolinic acids, by boiling with 50 ml. dilute H_2SO_4 ($\equiv$ 118·7 ml. N) in a stream of CO_2-free nitrogen, the gases evolved being passed up a reflux condenser and through a bubbler containing Brady's dinitrophenylhydrazine reagent, and then through a series of bubblers containing standardised baryta. After 3, 7, 14, 21 hours the amounts of CO_2 liberated corresponded to 73·0, 95·45, 129·45 and 138·85 ml. $N/10$ baryta respectively. Assuming $C_{10}H_{10}O_6 \rightarrow 2CO_2$, the theoretical amount of CO_2 is equivalent to 143·5 ml. $N/10$ baryta. One molecule of CO_2 was therefore readily eliminated but the second molecule only slowly. The dinitrophenylhydrazine bubbler showed a slight precipitate which increased on standing due to a little acetoin passing the condenser. The residual fluid after cooling required 119·2 ml. N alkali for neutralisation, corresponding to a residual acidity of 0·5 ml. N acid. On warming the neutral solution with excess N NaOH, a further amount of alkali was absorbed, owing to the hydrolysis and neutralisation of butyrolactone, making the total residual acidity up to 3·8 ml. N acid. Assuming that 1 mol. of carlic acid gives 1 mol. of butyrolactone, the theoretical amount of NaOH required is 3·6 ml. N. The neutralised solution was next evaporated *in vacuo* to dryness several times, the combined distillates made up to 500 ml. and investigated qualitatively and quantitatively (Wood-Ost method) for the presence of acetoin, whilst the salts remaining were acidified and the butyrolactone so obtained was isolated by continuous extraction with ether.

The distillates reduced Fehling's solution in the cold, gave a positive Voges-Proskauer reaction, gave slowly the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (not depressed by mixing with an authentic specimen) and gave a copper reduction by the Wood-Ost method corresponding to a total of 0·2767 g. acetoin (the theoretical amount assuming that 1 mol. of carlic acid gives 1 mol. of acetoin is 0·316 g.). The result is a little low, owing to the loss during the long period of bubbling, a loss detected in the dinitrophenylhydrazine bubbler.

The ether-extracted salts gave a sweet-smelling mobile oil which was identified as butyrolactone since it readily gave the beautifully crystalline phenylhydrazide of γ-hydroxybutyric acid, which crystallised in large plates from ethyl acetate, M.P. 94° (not depressed by mixing with an authentic specimen), prepared as described in our previous paper [Clutterbuck *et al.*, 1935].

Carlic acid (1 mol.) on hydrolysis with dilute mineral acid gives therefore 1 mol. each of acetoin and butyrolactone and 2 mols. of CO_2, one readily as with carolic acid, the second more slowly.

C. *Hydrolysis of carlosic acid.*

1·045 g. carlosic acid, M.P. 181°, were hydrolysed under the same conditions as with carlic acid by boiling with 40 ml. dilute H_2SO_4 ($\equiv$93·50 ml. *N*). After 3, 6, 13, 20 and 27 hours, the amounts of CO_2 liberated corresponded to 98·6, 126·0, 149·1, 165·5 and 171·7 ml. *N*/10 baryta respectively. Assuming $C_{10}H_{12}O_6 \rightarrow 2CO_2$, the theoretical amount of CO_2 is equivalent to 183·2 ml. *N*/10 baryta. One molecule of CO_2 was therefore readily eliminated and a second molecule more slowly. The dinitrophenylhydrazine bubbler again showed a precipitate due to a small amount of acetoin passing the condenser. The residual fluid after cooling required 97·90 ml. *N* NaOH for neutralisation, corresponding to a residual acidity of $97{\cdot}9-93{\cdot}5=4{\cdot}40$ ml. *N* acid. Assuming that 1 mol. of carlosic acid gives 1 mol. of a monobasic acid, the theoretical amount of residual acidity would be 4·58 ml. *N*. The neutralised solution was next evaporated *in vacuo* to dryness several times, the combined distillates made up to 500 ml. and investigated as before for the presence of acetoin, whilst the salts were acidified with 2*N* H_2SO_4, the hydrolysis acid separated by distillation in steam, neutralised and isolated as the *p*-phenylphenacyl ester by the method of Drake and Bronitsky [1930].

The distillates reduced Fehling's solution in the cold, gave a positive Voges-Proskauer reaction, gave slowly with Brady's reagent the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (not depressed on admixture with an authentic specimen), and gave a copper reduction by the Wood-Ost method corresponding to 0·3572 g. acetoin (the theoretical amount assuming that 1 mol. of carlosic acid gives 1 mol. of acetoin is 0·4035 g.). The *p*-phenylphenacyl ester of the volatile hydrolysis acid melted after recrystallisation from 95 % alcohol at 82°, and after fractional sublimation still melted at 82°. (Found: C, 76·39, 76·42; H, 6·30, 6·41 %. $C_{18}H_{18}O_3$ requires C, 76·58; H, 6·43 %.) A sample of the *p*-phenylphenacyl ester of *n*-butyric acid was synthesised and melted at 82° and did not depress the M.P. of the ester from the hydrolysis acid. It appears that Drake and Bronitsky's melting-point of 97° for the *p*-phenylphenacyl ester of *n*-butyric acid is incorrect. The *p*-phenylphenacyl ester of *iso*butyric acid was also prepared for comparison and melted both before and after sublimation at 89°. A mixture of this ester and that of the volatile acid from carlosic acid melted at 75–76°.

Carlosic acid (1 mol.) on hydrolysis with dilute mineral acid gives, therefore, 1 mol. each of acetoin and *n*-butyric acid and 2 mols. of CO_2, one readily as with carolic acid, the second more slowly.

D. *Reduction of carlic and carlosic acids.*

Carlic and carlosic acids are reduced readily under the same conditions as with carolic and carolinic acids.

Carlic acid (1 g.) was dissolved in 25 ml. of water, the catalyst prepared from 1 g. charcoal and 0·2 g. palladium chloride, as described for carolic acid, was added and the reduction started in the usual way; 2 mols. of hydrogen per molecule of carlic acid were absorbed in just over 1 hour. The catalyst was then filtered off and washed with a little NaOH and then with water, the filtrate and washings acidified and extracted several times with an equal volume of ether. After removal of the ether, a crystalline residue remained, which after recrystallisation from ether (containing a trace of alcohol)-light petroleum melted at 157·5°. (Found: C, 51·88, 51·92; H, 6·15, 6·09 %. $C_{10}H_{14}O_6$ requires C, 52·16; H, 6·13 %.)

0·1050 g. required 9·05 ml. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 116 ($C_{10}H_{14}O_6$ titrating as a dibasic acid requires 115).

It appears therefore that in the reduction of carlic acid $C_{10}H_{10}O_6$ (as with carolic acid), 2 mols. of hydrogen are absorbed and the reduced acid $C_{10}H_{14}O_6$ contains $2H_2$ more than carlic acid. This result is explained on the ketonic structure of carlic acid by assuming that on dissolving in water, 1 mol. of H_2O is first added in order to open the ring systems, and that the CO group of the side-chain is then reduced to CH_2, giving structure XIV.

Carlosic acid (1 g. in 90 ml. water) was reduced under exactly the same conditions as above. It absorbed 2 mols. of hydrogen in just over 1 hour. The reduced acid was almost insoluble in water and was precipitated during the reaction. The catalyst and acid were filtered off and extracted with NaOH, and the alkaline filtrate on acidification gave a crystalline precipitate which after recrystallisation from hot water melted at 217°. (Found: C, 56·01, 55·94; H, 6·61, 6·54 %. $C_{10}H_{14}O_5$ requires C, 56·06; H, 6·59 %.)

80·7 mg. required 7·50 ml. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 107·6 ($C_{10}H_{14}O_5$ titrating as a dibasic acid requires 107).

It appears therefore that in the reduction of carlosic acid $C_{10}H_{12}O_6$, 2 mols. of hydrogen are absorbed and that the product has the empirical formula $C_{10}H_{14}O_5$. This is readily explained by assuming that the CO group immediately outside the tetronic acid ring is reduced to CH_2, as in structure XV. The behaviour of carlosic acid on reduction is therefore completely analogous to that of carolinic acid.

The reduction of carlic and carlosic acids proceeds more slowly under our conditions than does the reduction of true unsaturated compounds, taking place at a rate almost identical with that of α-acetyltetronic acid, in which the CO group of the side-chain is reduced to CH_2 with production of α-ethyltetronic acid. The reductions of carlic and carlosic acids therefore support the ketonic structures assigned to these substances. This view, as will be shown in a subsequent publication, is strongly supported by the change of absorption spectrum on reduction, the double-banded spectra of carlic and carlosic acids giving way to single-banded spectra of the reduced acids. The behaviour of carlic and carlosic acids on reduction is therefore completely analogous to that of carolic and carolinic acids, respectively. This view is further supported by the fact that whereas carlic and carlosic acids give orange precipitates with $FeCl_3$, the corresponding reduced acids both give red colours with $FeCl_3$ indistinguishable from those given by γ-methyltetronic and α-ethyltetronic acids.

E. *Reactions of carlic and carlosic acids with 2:4-dinitrophenylhydrazine.*

Carlic acid, after standing with Brady's reagent either overnight or for a fortnight, gave no precipitate. The solution was extracted five times with ether and the ethereal solution extracted with aqueous $NaHCO_3$ when unchanged 2:4-dinitrophenylhydrazine remained in solution in the ether. The $NaHCO_3$ solution was acidified and re-extracted with ether. This ethereal solution on evaporation gave a red oil which could not be obtained crystalline. It appears therefore that carlic acid does react with the reagent, but the product is very soluble in water and has not been obtained in the crystalline condition. When a solution of carlic acid in Brady's reagent was allowed to stand for 8 weeks, a small amount of precipitate separated towards the end of this period. The precipitate proved however to be the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (unchanged on admixture with an authentic specimen), which must therefore have resulted from slow but complete hydrolytic degradation of carlic acid by the $2N$ HCl of the reagent.

An aqueous solution of carlosic acid gives with Brady's reagent an immediate orange-yellow precipitate. With strong aqueous solutions of carlosic acid, this precipitate consists of a mixture of the monodinitrophenylhydrazone of carlosic acid together with carlosic acid, the latter being only slightly soluble in the $2N$ HCl of Brady's reagent. After several recrystallisations from absolute ethyl alcohol, the monodinitrophenylhydrazone was obtained pure in orange-yellow needles, M.P. 182°. (Found (Schoeller) on a sample dried to constant weight at 128° *in vacuo*: C, 46·74; H, 3·97; N, 13·75 %. $C_{16}H_{16}O_9N_4$ requires C, 47·04; H, 3·95; N, 13·72 %.)

F. *Reactions of carlic and carlosic acids with diazomethane.*

The acids (1 g. in each case) were suspended in commercial (incompletely) dry ether and a solution of diazomethane in ether was added. With carlic acid, as with carolic acid, the reaction proceeded slowly for several hours. With carlosic acid as with carolinic, γ-methyltetronic and α-acetyltetronic acids, the reaction was vigorous and quickly complete. After standing overnight with excess of reagent, the ethereal solutions were evaporated and dried over H_2SO_4 *in vacuo* for several days. In both cases yellow oils resulted. With carlic acid the oil contained 24·1 % OMe ($C_{10}H_8O_4(OMe)_2 + H_2O$ requires 22·8 %), and with carlosic acid 27·2 % OMe ($C_{10}H_{10}O_4(OMe)_2$ requires 24·2 %).

The methylation products were then allowed to stand for 24 hours with 20 ml. N NaOH. The material in both cases passed completely into solution. After acidification and ether extraction, removal of solvent and drying, yellow oils were again obtained. The methoxyl content of the dimethyl derivative of carlic acid had fallen to 9·6 % and that of the dimethyl derivative of carlosic acid to 5·7 %.

The behaviour of carlosic acid and of the hydrated form of carlic acid on methylation with diazomethane, and of their dimethyl derivatives on treatment with cold dilute NaOH, is therefore strictly analogous to that of carolinic acid. The product of methylation in each case is a methyl ether methyl ester. In each case the carboxyl group is esterified and is relatively slowly hydrolysed, whereas the enolised carbonyl group in the β-position in the tetronic acid ring methylates to give a methyl ether which is readily hydrolysed, because there are two potentially acidic CO groups in β-positions to it.

The products resulting from the methylation with diazomethane of carlic and carlosic as well as carolic and carolinic acids all have methoxyl contents somewhat higher than the theoretical values, suggesting that the α-CO group of the side-chain is enolised to a slight extent and that the enolised portion is methylated.

SUMMARY.

Penicillium Charlesii G. Smith produces from glucose carlic acid ($C_{10}H_{10}O_6$) and carlosic acid ($C_{10}H_{12}O_6$). The molecular constitutions of these two acids have been investigated and it has been shown that they are closely related derivatives of *l*-γ-carboxymethyltetronic acid.

Carlic acid hydrate has been shown to be *l*-α-[γ-hydroxybutyryl]-γ-carboxymethyltetronic acid, and carlosic acid to be *l*-α-butyryl-γ-carboxymethyltetronic acid. Both acids are therefore closely related structurally to carolic and carolinic acids, which are also metabolic products of *P. Charlesii*, and to ascorbic acid (vitamin C).

A number of derivatives and breakdown products are described.

We gratefully acknowledge much assistance in the preparation of carlic and carlosic acids given by Messrs G. Smith and W. K. Anslow.

REFERENCES.

Benary (1911). *Ber. deutsch. chem. Ges.* **44**, 1763.

Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.

—— Raistrick and Reuter (1935). *Biochem. J.* **29**, 300.

Drake and Bronitsky (1930). *J. Amer. Chem. Soc.* **52**, 3715.

Ishikawa and Kojima (1934). *Sci. Rep. Tokyo Bunrika Daigaku*, Sect. A, I, 43–50 (*Chem. Zentr.* (1934), **1**, 2411).

CVI. ANTISCORBUTIC VALUE OF LUCERNE.

By LEOPOLD FERDINAND LEVY and FRANCIS WILLIAM FOX.

From the Biochemical Department, South African Institute for Medical Research, Johannesburg, South Africa.

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During the course of an investigation into the distribution of the antiscorbutic factor in some South African foodstuffs specimens of the common lucerne, or alfalfa (*Medicago sativa*) were examined, and it was found that, as judged by the indophenol reaction, this plant was an outstandingly rich source of vitamin C.

According to the report on the vitamins issued by the Medical Research Council [1932] lucerne is regarded as rich in vitamins A and B and exceptionally rich in E. More recent work, such as that described by Scheunert and Schieblich [1934] and by Hauge [1934], confirms the high values for vitamin A, though the former authors state that vitamins B and D are present only in low concentration. We have been unable, however, to find any reference dealing with the antiscorbutic value of this plant.

Lucerne is also of value since it contains no less than 1–2 % of mineral salts, of which nearly 0·5 % is calcium; in addition about 8 mg. of iron are present per g., or nearly double that found in spinach. It will be agreed, therefore, that lucerne is an interesting plant from the nutritional point of view; moreover, it is growing in favour as a forage crop and is becoming fairly readily available in many parts of the world [see monograph by Klinkowski, 1933]. In view of the foregoing it seemed desirable to investigate its antiscorbutic activity more closely.

Although it is well known that lucerne is occasionally the cause of death in animals, Steyn [1933], after careful examination of the available evidence is of the opinion that there is no experimental evidence to show that this is due to the presence of any poisonous substance in the plant. In all probability death is due to the effects of excessive fermentation, which, it appears, it likely to take place when large amounts are consumed, especially if the plant is in a young, frosted or wilted condition.

We learn from several sources that green lucerne is actually used to some small extent as a human food in parts of Russia, China and America, whilst here in South Africa it is occasionally used as a substitute for spinach. On account of its high mineral and vitamin A contents lucerne meal has been recently incorporated into the special "cereal mixture" devised by Brown and Tisdall [1933] and used by them with conspicuous success in the feeding of small children.

Determination of antiscorbutic activity by means of indophenol.

For this purpose the method of Birch *et al.* [1933] was used. The following are typical values from amongst many samples of lucerne that have been examined.

Table I. *Antiscorbutic value of lucerne as measured by the indophenol reaction.*

	mg. of ascorbic acid per g. of fresh lucerne
Young plants:	
Leaves only	3·2, 3·3, 3·3, 3·8, 3·8
Leaves and small stalks	2·7, 3·0, 3·1, 3·2, 3·2, 3·3
Shoots only	3·0, 3·8
Flowering stage:	
Leaves only	2·2, 2·2, 2·3, 2·3, 2·3, 2·4, 2·5, 2·6, 2·7, 2·7, 2·7, 2·7, 3·0, 3·1
Another specimen gave:	
Leaves only	4·5
Small stalks	1·8
Fibrous stalks	0·7

As will be seen from the above, the antiscorbutic value is of a high order, being frequently more than four times that of the same weight of orange juice.

Confirmation of antiscorbutic activity by means of animal experiments.

These experiments were carried out upon young guinea-pigs according to the method described by Bracewell *et al.* [1930]. A fresh supply of lucerne was obtained daily and the vitamin content estimated by means of indophenol. Only the leaves and young shoots were used, and the amounts weighed out and given to each animal were adjusted to correspond as nearly as possible to a definite quantity of vitamin C. The animals were divided into three groups, and the amounts of ascorbic acid given *per diem* corresponded approximately to 0·8, 1·6 and 3·2 mg. This represented a daily ration of 0·25–0·3, 0·5–0·6 and 1·0–1·2 g. respectively of the fresh leaf. A double quantity was given on Saturdays. At the conclusion of the experiment the animals were killed and

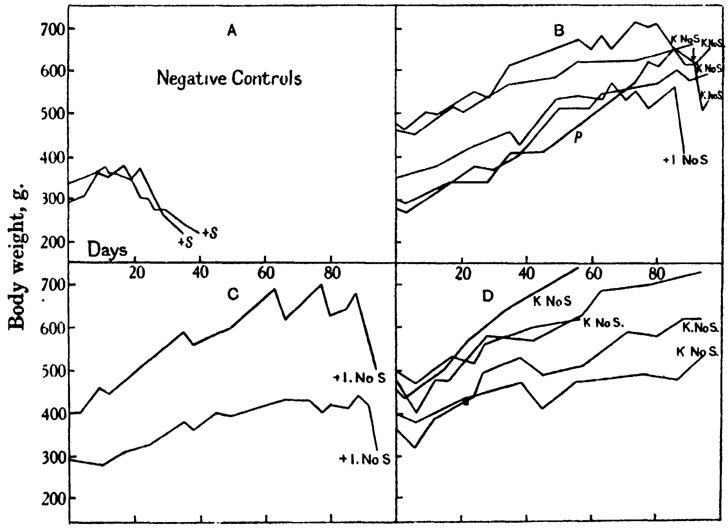

Fig. 1. A, Negative controls; B, 0·25–0·3 g. lucerne≡0·8 mg. ascorbic acid; C, 0·5–0·6 g. lucerne≡1·6 mg. ascorbic acid; D, 1·0–1·2 g.≡3·2 ascorbic acid.

+S Died of scurvy.
+I. Died of intercurrent infection.
K. Killed.
No S. No scurvy.
P Pregnant.
↓ Birth of young.

carefully examined for evidence of scurvy. No such evidence was obtained from any of the animals receiving the lucerne, whereas both the animals receiving the basal diet alone quickly died from this disease. These two animals showed extensive subcutaneous haemorrhages, fragile epiphyses containing numerous areas of haemorrhage in the marrow and beading of the costochondral junctions; the teeth, however, did not appear to be brittle nor was there swelling or haemorrhage into the gums. The charts of the animals' growth are given in Fig. 1.

Some difficulty arises in the quantitative interpretation of these animal experiments, owing to the uncertainty which appears to exist regarding the minimum protective dose for the guinea-pig. According to Birch *et al.* [1933] 0·9 mg. of ascorbic acid *per diem* may be regarded as sufficient to protect the animal from macroscopical evidence of scurvy, whilst Göthlin [1934] claims that protection against macroscopical scorbutic alterations can be reached with values as low as 0·5 mg. of ascorbic acid. Since 0·25–0·3 g. of lucerne leaf *per diem* protected these animals from macroscopical evidence of scurvy, it may be concluded that this amount of leaf contains at least 0·5 mg. of the vitamin. This corresponds to 2·0–3·6 mg. per g. of leaf depending on which value for the minimum protective dose is accepted; in either case the values obtained by the chemical and biological methods are seen to be in very fair agreement.

Variations in the antiscorbutic activity of lucerne.

As will be noticed from Table I, the shoots and leaves tend to be somewhat richer when young than at the flowering stage. There are a very great many different varieties of lucerne in cultivation [see Klinkowski, 1933], and it is quite possible that these varieties may differ very considerably in their antiscorbutic values and in other properties that may be of importance when considering lucerne as a possible green vegetable for human consumption. The variety used for the above experiments is known as Provence; substantially the same values were, however, obtained for a few specimens of two other varieties in common use, namely Chinese and Hunter's River. No difference was found between samples taken at the same time from a variety receiving different fertiliser treatment.

Preservation of the antiscorbutic value of lucerne.

Green lucerne. The changes that are liable to occur after cutting are seen in Table II. In the first case there is an apparent increase in concentration owing to wilting of the leaves, whilst in the second, although wilting was prevented, the lucerne became slightly yellow in colour; in such cases lower values were invariably obtained.

Table II. *Deterioration of antiscorbutic value of lucerne after cutting.*

mg. ascorbic acid per g. of leaf.

	Standing without water	Standing in water
Fresh leaves	3·2, 3·2, 3·3	3·1, 2·6, 2·5, 3·0
After 24 hours	3·8, 3·7, 3·6	2·3, 2·2, 2·3, —
After 72 hours	— — —	1·9 — — 2·1

That lucerne, when cooked like spinach, retains its antiscorbutic activity to a very considerable extent is suggested by the following experiment.

10·6 g. of carefully sampled lucerne leaves, corresponding to an ascorbic acid content of 34·0 mg., were boiled for 20 minutes with 50 ml. of water and a pinch of salt in a covered pyrex beaker. At the conclusion of the experiment very little water remained, and after the contents were cooled 31·7 mg. or 90 % of the original ascorbic acid was found to be present.

Lucerne hay. That ordinary lucerne hay of commerce may contain a good deal of ascorbic acid is seen from the following figures obtained for samples of varying quality found at the local market:

0·09, 0·28, 0·45 and 1·6 mg. per g. of hay.

Lucerne meal. Specimens of commercial meal were also examined and were found to vary considerably in their ascorbic acid contents. Two samples contained negligible amounts, whilst the best, which was of a good green colour and contained much less stalk, gave a value of 0·57 mg. per g.

A sample of meal was also prepared from a bale of lucerne obtained from the market; the leaves when removed from the stalks were found to represent about 37 % of the original weight. On grinding the meal gave a value of 0·79 mg. per g.

It was thought that the rate of destruction of the vitamin during the curing of green lucerne and the preparation of meal would be much reduced if enzyme action could be arrested at an early stage. The following rough experiments support this view and indicate possible methods for the practical preparation of meals possessing really high antiscorbutic activity.

(1) Fresh lucerne plants were plunged into boiling water for 30 seconds and then dried by exposure to air, but away from direct sunlight.

Table III. *Lucerne meal prepared from leaves scalded for 30 seconds.*

mg. ascorbic acid per g.

	Initial value		Value after scalding and drying	
	Fresh leaves	Calculated to dry weight	4 days' drying	11 weeks' storage
(*a*)	—	—	1·5	—
(*b*)	3·1	10·3	3·8	—
(*c*)	2·4	8·0	4·4	—
(*d*)	3·1	10·3	4·0	2·7

(2) Lucerne dried in an air-oven for 6 minutes at 250° gave values of over 7·0 mg. per g.

(3) Lucerne leaves and shoots were dried for 2 days over sulphuric acid *in vacuo*. The excellent preservation obtained is seen from Table IV.

Table IV. *Antiscorbutic value of desiccated lucerne.*

Initial value		Value after desiccation	
Fresh leaves	Calculated to dry weight	Immediately after desiccation	After 6 months' storage
2·3	9·2	8·3	—
2·5	10·0	7·4	3·0

These three experiments indicate that there should be no great difficulty in devising a practical method for the preparation of a lucerne meal possessing considerable antiscorbutic activity, should such a dry and comparatively non-perishable source of the vitamin be required.

SUMMARY.

1. Lucerne is an outstandingly good source of vitamin C, the concentration in the average leaf examined being about four times that found in *Citrus* juice. This conclusion was first arrived at by means of the indophenol reaction and then confirmed by the biological test.

2. Although the original activity rapidly diminishes when the cells are damaged, preliminary experiments indicate that much of the vitamin remains after cooking, and that it is possible to dry the plant in such a way that lucerne meal of high activity can be obtained.

3. Since lucerne is also a rich source of vitamins A and E and is also unusually rich in minerals such as calcium and iron, the possibility of using certain types of the young plant as a human food, either as green salad, spinach or in the form of dry meal is worthy of consideration.

We should like to take this opportunity of thanking Dr F. W. Simson, Pathologist to the Institute, for his help with the *post mortem* examination of the animals, Dr J. Daneel, Superintendent of the Rietfontein Hospital for arranging the daily supplies of freshly gathered lucerne, and Miss C. Wilson, of the Sanitation Department, Rand Mines, for suggesting and carrying out the experiment with oven-dried lucerne.

REFERENCES.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Bracewell, Hoyle and Zilva (1930). *Med. Res. Coun. Sp. Rep.* No. 146.
Brown and Tisdall (1933). *Brit. Med. J.* i, 55.
Göthlin (1934). *Nature*, **134**, 569.
Hauge (1934). *J. Offic. Agric. Chem.* **17**, 304.
Klinkowski (1933). *Bull. No.* **12**, *Imp. Bur. Plant Genetics, Aberystwyth.*
Medical Research Council (1932). *Spec. Rep. Ser.* No. 167.
Scheunert and Schieblich (1934). *Zentr. Tierernährung*, **6**, 112 (*Nutrit. Abstr.* 1934, **4**, 267).
Steyn (1933). Toxicology of plants in South Africa. (London Central News Agency.)

CVII. STUDIES IN THE METABOLISM OF THE STRICT ANAEROBES (GENUS *CLOSTRIDIUM*).

III. THE OXIDATION OF ALANINE BY *CL. SPOROGENES*.

IV. THE REDUCTION OF GLYCINE BY *CL. SPOROGENES*.

By LEONARD HUBERT STICKLAND[1].

From the Biochemical Laboratory, Cambridge.

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In the first paper of this series [Stickland, 1934] it was shown that washed suspensions of *Cl. sporogenes* can catalyse reactions between various pairs of amino-acids, one amino-acid being oxidised and the other reduced. In the second paper [Stickland, 1935] the reduction of proline was shown to occur by rupture of the ring, giving δ-aminovaleric acid. The present work continues the study of the chemistry of these reactions and deals with the oxidation of alanine and the reduction of glycine.

III. THE OXIDATION OF ALANINE BY *CL. SPOROGENES*.

Experimental.

Preparation of bacterial suspensions.

The culture was the same as that previously used (*Cl. sporogenes* Bellette), and the washed suspensions of bacteria were prepared in the way described in the earlier papers.

The oxidation of alanine.

(*a*) *The degree of oxidation of alanine.* In the first paper it was proved that when alanine is oxidised by cresyl blue two molecules of the dye are reduced for each molecule of alanine oxidised. This result has now been confirmed, using the natural substrate glycine in place of the artificial dye. A series of Thunberg tubes was set up each containing a known amount of *d*-alanine (0·5 ml. of $M/20$ solution), excess of glycine (0·5 ml. of $M/5$ or 0·5 ml. of $M/2{\cdot}5$ solution), 0·5 ml. of phosphate buffer $M/2$ p_H 7·5 and 1·0 ml. of suspension of bacteria. A control series of tubes contained the same solutions with 0·5 ml. of water replacing the alanine solution. After evacuation and incubation at 40°, pairs of tubes were taken and the ammonia was estimated in 2 ml. of their contents, estimations being carried out from time to time until no further increase in the ammonia (corrected for the blank) was found. Since only the final value was required, samples were usually not taken until after 24 hours, when the reaction was practically complete. The result of one experiment is given in Table I. The ammonia production approaches a limit at 0·74 ml. of $M/10$ per tube. Of this 0·25 ml. is derived from the oxidation of the alanine, so that 0·49 ml. can be attributed to reduction of glycine. Hence 2·0 molecules of glycine are reduced for each molecule of alanine oxidised; two other similar experiments gave values

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[1] Beit Memorial Fellow.

Table I.

Time of incubation hours	Ammonia found per tube (corrected for control) ml. $M/10$
22	0·66
34	0·71
47	0·74

of 1·8 and 1·8. Making the assumption, which will be justified experimentally later on, that the reduction of glycine involves the taking up of two equivalents of hydrogen, this result agrees with that obtained by the oxidation of alanine with cresyl blue.

In connection with these experiments it must be mentioned that when thick suspensions of bacteria are used, and the experimental period is rather long, a significant amount of ammonia is produced in tubes containing bacteria and glycine alone, in excess of that found in the blank. This must be attributed to the reduction of a small amount of glycine by the "reducing blank" of the bacteria. This point was already apparent even in the shorter experiments in the first paper of this series (see some results in Table V of that paper [Stickland, 1934, p. 1754]).

(*b*) *The products of oxidation of alanine.* The product of reduction of proline has already been identified as δ-aminovaleric acid [Stickland, 1935], so that from an examination of the products of the reaction between one molecule of alanine and two molecules of proline the oxidation products of alanine can be found. Preliminary experiments showed that ammonia, carbon dioxide and a volatile acid were among the compounds formed.

For estimation of the volatile acid, 10 ml. of $M/10$ *d*-alanine solution, 10 ml. of $M/5$ proline, 10 ml. of $M/2$ phosphate buffer p_H 7·5 and 20 ml. of suspension of *Cl. sporogenes* were placed in a large (75 ml.) Thunberg tube, which was evacuated and incubated at 40°. A control tube with 20 ml. of water in place of the two amino-acid solutions was treated similarly. For carbon dioxide estimation, a 50 ml. bottle containing 2·5 ml. of $M/10$ alanine, 2·5 ml. $M/5$ proline, 2·5 ml. buffer and 5·0 ml. bacterial suspension was connected in the incubator with a train of three small gas wash-bottles, each containing 5 ml. of $N/10$ baryta and rendered anaerobic by the passage of a stream of oxygen-free nitrogen. A parallel control with neither amino-acid was set up at the same time. A Kjeldahl splash trap was inserted between the experimental bottles and the baryta wash-bottles and the rubber connections were made as short as possible and coated with paraffin wax. The course of the reaction was followed by preparing two sets of small Thunberg tubes containing the solutions in the same proportions as above in a total volume of 2·5 ml. (0·5 ml. $M/10$ alanine, 0·5 ml. $M/5$ proline, 0·5 ml. buffer and 1·0 ml. bacteria, and the corresponding controls) in which the ammonia was estimated from time to time until the whole of the alanine was found to be oxidised.

The reaction was usually complete in from 12 to 24 hours, after which the estimations were carried out.

For the carbon dioxide estimations, the current of nitrogen was increased and allowed to run for 2 hours or more. This was found by trial to be sufficient to remove the whole of the carbon dioxide from the solution even at p_H 7·5. The baryta was then titrated with $N/10$ hydrochloric acid to phenolphthalein.

For volatile acid estimations, 40 ml. of the contents of each large Thunberg tube were freed from chloride by adding them to a mixture of 10 ml. of N sulphuric acid and 40 ml. of saturated silver sulphate solution. Volatile acid was estimated in duplicate on 40 ml. portions of the filtrates by distilling *in vacuo* into excess of standard baryta. The manipulations of the acid solutions sufficed to remove the carbon dioxide without further treatment, as was shown by the fact that the baryta in the receiver remained free from barium carbonate.

Ammonia was estimated in duplicate in 2 ml. portions of the residues from both the carbon dioxide and the volatile acid experiments; these two values were always identical, within experimental error.

The complete results of two experiments are given in Table II. Since the product of reduction of proline (δ-aminovaleric acid) is not volatile, all these products must have arisen from the oxidation of alanine, and the figures though not exact show that by far the greater part of the alanine has been oxidised to one molecule of ammonia, one of a volatile acid and one of carbon dioxide from each molecule of alanine oxidised. (The possibility that the acid titration represents half a molecule of a dibasic acid need not be considered, as all the simpler volatile acids are monobasic.)

Table II. *Products of the reaction between 10 ml.* M/*10* d-*alanine and 10 ml.* M/*5 proline.*

Expressed as ml. *M*/10.

	Exp. 1			Exp. 2		
	Exp.	Control	Diff.	Exp.	Control	Diff.
Ammonia	9·5	0·5	9·0	9·8	0·4	9·4
Volatile acid	9·4	1·1	8·3	9·7	1·2	8·5
Carbon dioxide	10·4	0·8	9·6	8·1	0·3	7·8

(*c*) *The identity of the volatile acid.* (1) *By the* p-*toluidide.* The identification of a volatile acid is best effected by the formation of the *p*-toluidide. The yield of this derivative by the standard method is however very small, and it has proved impossible to obtain enough of the acid formed by oxidation of alanine by proline to identify it in this way.

(2) *By the lanthanum-iodine reaction.* Krüger and Tschirch [1929; 1930] described a test for acetate depending on the formation of a solution of basic lanthanum acetate, which gives a blue colour with iodine similar to that obtained with starch. The test is specific for acetate and propionate, but is modified or inhibited by many other anions. It is given strongly by the neutralised distillates from the oxidation products of alanine. When the test is carried out at room temperature, it is found that the depth of colour is by no means proportional to the concentration of acetate present but falls off very sharply between acetate concentrations of *M*/100 and *M*/500. (The original authors claim that by gradually heating the mixed solutions a positive reaction may be obtained down to *M*/6000 acetate, but the present author has not been able to detect less than *M*/1000 under any conditions.) The blue colour cannot therefore be used in the ordinary way for colorimetric estimation of acetic acid, but the sharp decrease in intensity between *M*/100 and *M*/500 may be used as the basis of a method of approximate estimation, in the following manner.

The acetic acid to be estimated is distilled over into excess of alkali, to free it from sulphate and phosphate which inhibit the reaction. The distillate is neutralised, evaporated if necessary and made up to such a volume that it contains roughly *M*/50 acetate (the correct dilution must be determined by trial). Into a series of test-tubes are measured volumes of this solution from 1·0 ml. down to 0·1 ml., and each is made up to 1 ml. Into another series of test-tubes are measured similar volumes of a standard $M/50$ solution of sodium acetate. To each of these tubes are now added 0·5 ml. of 5 % lanthanum nitrate solution, 0·5 ml. of $N/100$ alcoholic solution of iodine and three or four drops of N ammonia. From a comparison of the blue colours developed in the two series of tubes it is possible to deduce with some accuracy (probably ± 10 %) the concentration of acetate in the unknown solution.

This method was applied to the volatile acid distillates obtained from experiments on the oxidation of alanine by proline. The distillates after titration to neutrality were evaporated and made up as accurately as possible in a measuring cylinder to a volume corresponding to that which they had occupied in the original experimental tube (*viz.* 17·7 ml.). If the whole of the volatile acid were acetic acid, the concentration of acetate in this solution should now be $M/50$. The lanthanum-iodine test was carried out on a series of dilutions of this solution and simultaneously on a similar series of dilutions of a $M/50$ solution of sodium acetate. The details and results of two experiments are given in Table III. These figures show that, within the rather wide limits of experimental error, the whole of the volatile acid is acetic acid, since propionic acid, which also gives the test, is obviously impossible. The equation representing the oxidation of alanine can therefore be written

Table III.

Volume of standard or unknown ml.	Blue colour			
	Standard acetate	Alanine + 2 proline exp.	Alanine + 2 proline exp.	Control exp.
1·0	+ + + + + +	+ + + + +	+ + + + + +	–
0·5	+ + + + +	+ + + +	+ + + + +	–
0·4	+ + + +	+ + +	+ + + +	–
0·3	+ + +	+ +	+ +	–
0·2	+ +	(+)	+	–
0·1	–	–	–	–
Estimated concentration of acetate		0·015 M	0·018 M	Nil

$$CH_3.CHNH_2.COOH-4H+2H_2O \rightarrow CH_3.COOH+NH_3+CO_2.$$

(*d*) *Intermediate products in the oxidation of alanine to acetic acid.* Though it is possible that this oxidation proceeds in a single step of four equivalents, it is far more likely to involve two steps of two equivalents each. The most probable course appears to be

$$(1)\ CH_3.CHNH_2.COOH-2H+H_2O \rightarrow CH_3.CO.COOH+NH_3,$$
$$(2)\ CH_3.CO.COOH-2H+H_2O \rightarrow CH_3.COOH+CO_2,$$

pyruvic acid being an intermediate compound. Experiments already quoted [Stickland, 1934] showed that pyruvic acid could not be detected at any stage of the reaction, whether the oxidant were cresyl blue, proline or glycine, within the limits of sensitivity of the nitroprusside test. Therefore if pyruvic acid is indeed the intermediate compound it must be oxidised as quickly as it is formed so that it does not accumulate in the solution. Direct proof of the occurrence of pyruvic acid has not been obtained, but evidence supporting this view has been obtained by proof of the following points:

(1) The rate of oxidation of the intermediate product is greater than that of alanine.

(2) The rate of oxidation of pyruvic acid is greater than that of alanine.

(3) The extent of oxidation of pyruvic acid is in agreement with equation (2) above.

(4) The products of oxidation of pyruvic acid are in agreement with equation (2).

(1) *The rate of oxidation of the intermediate compound.* If we consider the reaction between one molecule of alanine and one of proline, it is clear that (*a*) if the intermediate compound is oxidised at a negligible speed, then the whole of the alanine will be oxidised to this compound, and all its nitrogen will be liberated as ammonia; (*b*) if the intermediate product is oxidised very rapidly, then the proline will suffice for the complete oxidation of only one-half of the alanine, and only 50 % of the nitrogen will appear as ammonia. Thus the quantity of ammonia liberated in this reaction serves to indicate the relative velocities of oxidation of alanine and the hypothetical intermediate compound. To test this experimentally, three sets of Thunberg tubes were prepared containing (*a*) 0·5 ml. $M/10$ alanine, 0·5 ml. $M/10$ proline, 0·5 ml. phosphate buffer p_H 7·5 and 1·0 ml. suspension of *Cl. sporogenes*, (*b*) 0·5 ml. $M/10$ alanine, 0·5 ml. $M/5$ proline, 0·5 ml. buffer and 1·0 ml. suspension and (*c*) 1·0 ml. water, 0·5 ml. buffer and 1·0 ml. suspension. After anaerobic incubation at 40° for various periods, the ammonia was estimated in 2 ml. of the contents of one tube from each series, and these estimations were continued until the ammonia produced, corrected for the blank, remained constant. A typical result is shown in Fig. 1.

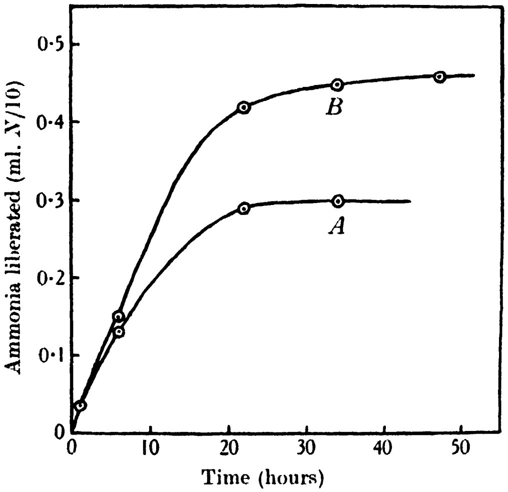

Fig. 1. The course of production of ammonia from: *A*. 0·5 ml. $M/10$ alanine + 0·5 ml. $M/10$ proline; *B*. 0·5 ml. $M/10$ alanine + 0·5 ml. $M/5$ proline.

In this case, while the reaction of alanine with two molecules of proline liberated as usual practically the whole of nitrogen of the alanine as ammonia, the reaction with only one molecule of proline gave only slightly more than half (60 %). Other similar experiments gave values of 58, 57, 68, 57, 66 and 60 % (average 61 %). It can therefore be concluded that if an intermediate compound is formed in the oxidation of alanine it is oxidised more rapidly than alanine itself.

(2) *The rate of oxidation of pyruvic acid.* In the first paper [Stickland, 1934] it was shown that, when cresyl blue is the oxidant, pyruvic acid is oxidised with a velocity about 40 % of that of the oxidation of alanine. Comparison of the rates of oxidation of alanine and pyruvic acid by glycine, however, showed that

in this case the latter is oxidised more rapidly than the former (Table IV). The rates were measured as usual by estimation of the rate of liberation of ammonia from the glycine; allowance is made for the fact that in the alanine oxidation ammonia is formed from both reactants, in the pyruvate from only one.

Table IV.

0·5 ml. $M/10$ glycine +0·5 ml. $M/10$	Ammonia produced in 5 hrs. (ml. $M/10$)	Rate of oxidation (ml. $M/10$ per hr.)	$\frac{\text{Rate of oxidation of pyruvate}}{\text{Rate of oxidation of alanine}}$
Alanine	0·32	0·021	2·1
Pyruvate	0·22	0·044	
Alanine	0·32	0·021	2·3
Pyruvate	0·24	0·048	

(3) *The extent of oxidation of pyruvate.* The extent of oxidation of pyruvic acid was measured by incubating a known amount of pyruvate with excess of glycine and determining the amount of ammonia liberated by reduction of the glycine, exactly as in the case of alanine.

Commercial pyruvic acid was distilled *in vacuo*, the fraction boiling at about 66°/15 mm. being collected. This was diluted with ice-cold water to give a roughly 10 % solution and stored in the ice-chest. Titration with alkali and titration of bisulphite-binding capacity agreed exactly, showing the product to be pure. For use in experiments portions of this solution were carefully neutralised immediately before use and diluted to $M/10$.

To measure the degree of oxidation of pyruvic acid, 0·5 ml. $M/10$ or $M/20$ sodium pyruvate solution, 0·5 ml. $M/2{\cdot}5$ or $M/5$ glycine, 0·5 ml. buffer of p_{H} 7·5 and 1·0 ml. of suspension of bacteria were placed in each of five Thunberg tubes, and in another five tubes the same solutions with 0·5 ml. of water replacing the pyruvate solution. Estimations of ammonia in experimental tubes and controls were carried out from time to time until no further increase was observed. The corrected value for the final ammonia produced showed that for each molecule of pyruvate oxidised one molecule of glycine was reduced (see Table V). This is the degree of oxidation required for the oxidation of pyruvate to acetate as in equation (2) on p. 892.

Table V. *Extent of oxidation of pyruvate.*

Pyruvate	Glycine	NH_3 corrected for blank (ml. $M/10$)	$\frac{\text{Mols. glycine reduced}}{\text{Mols. pyruvate oxidised}}$
0·5 ml. $M/10$	0·5 ml. $M/5$	0·39	0·8
0·5 ml. $M/20$	0·5 ml. $M/5$	0·21	0·8
0·5 ml. $M/20$	0·5 ml. $M/2{\cdot}5$	0·23	0·9

(4) *The products of oxidation of pyruvate.* The products of oxidation of pyruvic acid were determined exactly as in the case of alanine, by means of the reaction with proline. For estimation of volatile acid, two 75 ml. Thunberg tubes were prepared containing (*a*) 10 ml. $M/10$ sodium pyruvate, 10 ml. $M/10$ proline, 10 ml. $M/2$ buffer p_{H} 7·5 and 20 ml. suspension of bacteria and (*b*) 20 ml. water, 10 ml. buffer and 20 ml. suspension. These were evacuated and incubated at 40°. For carbon dioxide estimation two 50 ml. bottles, containing one-quarter of the above quantities of the solutions, were connected in the incubator to a series of three small wash-bottles each containing 5 ml. of $N/10$ baryta. These were rendered anaerobic by the passage of a current of oxygen-free nitrogen, with the precautions mentioned in the alanine experiment. The reaction could

obviously not be followed by estimation of the ammonia produced, so instead a series of small Thunberg tubes was set up with the same solutions in a total volume of 2·5 ml. (no controls were necessary), and the disappearance of the pyruvic acid followed by means of the nitroprusside test (2 % of the initial concentration gave a positive test, so disappearance of the test indicated that more than 98 % of the pyruvate had reacted). When the reaction was finished, the estimations of ammonia, volatile acid and carbon dioxide were carried out as before. The results of two such experiments are given in Table VI and are sufficient to show that from each molecule of pyruvic acid there arise one molecule of volatile acid and one of carbon dioxide.

Table VI. *Products of the reaction between 10 ml.* M/*10 Na pyruvate and 10 ml.* M/*10 proline.*

Results expressed as ml. *M*/10.

	Exp. 1			Exp. 2		
	Exp.	Control	Diff.	Exp.	Control	Diff.
Ammonia	0·5	0·6	0·0	0·6	0·5	0·1
Volatile acid	13·0	2·8	10·2	11·7	2·7	9·0
Carbon dioxide	8·2	0·3	7·9	10·7	0·7	10·0

The volatile acid was identified and roughly estimated by means of the reaction of Krüger and Tschirch [1929], in exactly the same manner as that arising from alanine. The result is given in Table VII, and the figures show that within the experimental error all the volatile acid is acetic acid (propionic acid may again obviously be excluded from consideration). This confirms the equation representing the oxidation of pyruvic acid given on p. 892.

Table VII.

Volume of standard or unknown ml.	Blue colour		
	Standard acetate	Pyruvate + proline exp.	Control exp.
1·0	+ + + + + +	+ + + + + +	–
0·5	+ + + + +	+ + + +	–
0·4	+ + + +	+ + +	–
0·3	+ + +	+ +	–
0·2	+ +	+	–
0·1	–	–	–
Estimated concentration of acetate		0·018 *M*	Nil

The reduction of methylviologen by alanine.

In the first of these papers it was pointed out that the calculations of Wurmser and Mayer-Reich [1933] indicated that an alanine dehydrogenase system whose end-product was ammonium pyruvate should have a potential in the neighbourhood of r_H 12, while the alanine dehydrogenase of *Cl. sporogenes* actually reduced dyes down to about r_H 2 (methylviologen at p_H 8·0). This discrepancy can now be explained.

A concentration of *M*/50 *d*-alanine at p_H 7·5 gives about 10 % reduction of methylviologen. It was found on further investigation that sodium pyruvate under exactly the same conditions gave a much greater reduction, *viz.* about 70 %. The probable explanation of the reduction in the presence of alanine is that as soon as a trace of oxidation to pyruvate takes place, this product is further oxidised to acetate, and it is this second reaction which produces the measurable amount of reduction of the indicator. The final equilibrium reached, starting from alanine and methyl viologen, is a very complex one consisting of alanine-NH_4-pyruvate-acetate-bicarbonate-methylviologen-leucomethylviologen, and the quantitative relationships in a case like this have not yet been worked out, but it is evident that a qualitative explanation of the discrepancy mentioned above has been provided.

DISCUSSION.

The end-products of the oxidation of alanine by proline in presence of the enzymes of *Cl. sporogenes* are clearly ammonia, acetic acid and carbon dioxide. Attempts to show directly that pyruvic acid occurs as an intermediate stage in the oxidation have failed; for instance, the addition of sodium arsenite, used successfully by Krebs [1933] for this purpose in the case of mammalian tissue slices, completely inhibited the whole reaction. The indirect evidence obtained, however, is all in favour of pyruvic acid as an intermediate product, and it may be considered as extremely probable that the reaction occurs in the stages given in the equations on p. 892.

IV. THE REDUCTION OF GLYCINE BY *CL. SPOROGENES*.

EXPERIMENTAL.

Estimation of the products of reduction of glycine.

As the products of oxidation of alanine have already been shown to be acetic acid, carbon dioxide and ammonia, the determination of the products of the reaction between alanine and glycine would suffice to decide the fate of the glycine. Experiments were therefore carried out in which one molecule of alanine was oxidised by two molecules of glycine. The experiments were set up as before in three sections: (*a*) for volatile acid estimations, a 75 ml. Thunberg tube containing 10 ml. $M/10$ *d*-alanine, 10 ml. $M/5$ glycine, 10 ml. $M/2$ phosphate buffer p_H 7·5 and 20 ml. bacterial suspension, (*b*) for carbon dioxide estimation, a 50 ml. bottle containing 2·5 ml. $M/10$ alanine, 2·5 ml. $M/5$ glycine, 2·5 ml. buffer and 5 ml. suspension, and (*c*) for following the course of the reaction, a set of five 10 ml. Thunberg tubes each containing 0·5 ml. alanine, 0·5 ml. glycine, 0·5 ml. buffer and 1·0 ml. suspension. In each case control experiments were also prepared, differing by the omission of both amino-acids. The Thunberg tubes in (*a*) and (*c*) were evacuated in the usual way and incubated at 40°. The bottles in (*b*) were connected in the incubator at 40° to a series of three wash-bottles, each containing 5 ml. of $N/10$ barium hydroxide and rendered anaerobic by the passage of a stream of pure nitrogen, with the precautions mentioned in the previous paper. The reaction was followed by estimation of the ammonia in 2 ml. of the contents of the small Thunberg tubes (*c*), and incubation was continued until the quantity of ammonia liberated, corrected for the control, approached the theoretical for the complete reaction. In most experiments the reaction started immediately, was almost complete (about 90 %) in 24 hours and had reached 97–98 % of the theoretical value in 48 hours. Volatile acid was estimated in the contents of the large Thunberg tubes, after removal of the chloride with silver sulphate, by distillation *in vacuo* into standard barium hydroxide, and carbon dioxide by titration of the barium hydroxide in the wash-bottles, as described in the first part of this paper.

Ammonia was estimated in both the volatile acid experiment (*a*) and the carbon dioxide experiment (*b*), always with concordant results. The results of two experiments are given in Table I. The figures show that when one molecule of alanine is oxidised by two molecules of glycine, there arise three molecules of ammonia, one of carbon dioxide and three of volatile acid. Of these products, one molecule of ammonia, one of carbon dioxide and one of volatile acid (acetic) proceed from the oxidation of the alanine, so that two molecules each of ammonia and of a volatile acid must be the products of the reduction of two molecules of glycine.

Table I. *Products of the reaction between 10 ml.* M/10 d-*alanine and 10 ml.* M/5 *glycine.*

Results expressed as ml. $M/10$.

	Exp. 1			Exp. 2		
	Exp.	Control	Diff.	Exp.	Control	Diff.
Ammonia	31·8	2·6	29·2	30·6	1·2	29·4
Carbon dioxide	10·1	1·4	8·7	10·5	0·7	9·8
Volatile acid	30·8	1·8	29·0	30·5	3·7	26·8

The identification of the volatile acid.

(*a*) *By the* p-*toluidide.* The acid distillates from one experiment were collected, evaporated to dryness and dried at 110°. The solid was heated with *p*-toluidine and a little strong hydrochloric acid in the usual way, and from the mixture about 25 mg. of a toluidide were separated. After two recrystallisations from hot water, only a few milligrams of a still slightly impure product remained, and with this a melting-point determination was made and a mixed melting-point with some pure acet-*p*-toluidide. The figures obtained were:

	Melting-point
Acet-*p*-toluidide	147°
Product from action of bacteria	145°
Mixture	146°

These are sufficient to indicate that the volatile acid consisted at any rate chiefly of acetic acid.

(*b*) *By the lanthanum-iodine test.* The acid was identified and roughly estimated by the method already described, based on the reaction of Krüger and Tschirch [1929]. Table II contains the results of one such experiment, with the results of an experiment on the oxidation of alanine by proline for comparison. The results show that, within the rather large limits of experimental error, all the volatile acid produced is acetic acid.

Table II.

ml. of $M/50$ sodium acetate or of unknown solution	Blue colour produced			
	Standard acetate	Alanine + 2 proline	Alanine + 2 glycine	Control
1·0	+ + + + + +	+ + + + + +	+ + + + + +	–
0·5	+ + + + +	+ + + + +	+ + + + + +	–
0·4	+ + + +	+ + + +	+ + + + + +	–
0·3	+ + +	+ +	+ + + + +	–
0·2	+ +	+	+ + + +	–
0·1	–	–	+ +	–
Estimated acetate concentration	(0·02 M)	0·018 M	0·055 M	Nil

CONCLUSION.

The formation of one molecule of ammonia and one of a volatile acid from each molecule of glycine reduced, and the identification of the acid as acetic acid, show that the reduction of glycine can be expressed by the following equation:

$$CH_2NH_2COOH + 2H \rightarrow CH_3COOH + NH_3.$$

The formation of acetic acid from glycine by the action of mixed cultures of putrefactive bacteria has already been demonstrated by Brasch [1909].

SUMMARY.

When alanine is oxidised by *Cl. sporogenes*, at the expense of the reduction of proline or glycine, it loses four equivalents of hydrogen and yields one molecule each of acetic acid, carbon dioxide and ammonia.

The reaction probably proceeds in two stages of two equivalents each, ammonium pyruvate being the intermediate product.

Washed suspensions of *Cl. sporogenes* reduce glycine, at the expense of the oxidation of alanine, to acetic acid and ammonia.

REFERENCES.

Brasch (1909). *Biochem. Z.* **22**, 403.
Krebs (1933). *Z. physiol. Chem.* **217**, 191.
Krüger and Tschirch (1929). *Ber. deutsch. chem. Ges.* **62**, 2776.
—— —— (1930). *Ber. deutsch. chem. Ges.* **63**, 826.
Stickland (1934). *Biochem. J.* **28**, 1746.
—— (1935). *Biochem. J.* **29**, 288.
Wurmser and Mayer-Reich (1933). *Compt. Rend. Soc. Biol.* **112**, 1648.

CVIII. THE ABSORPTION SPECTRA OF ACIDS FROM FISH-LIVER OILS[1].

By JOSEPH RATCLIFFE EDISBURY
AND RICHARD ALAN MORTON,
Department of Chemistry, University of Liverpool,
AND JOHN ARNOLD LOVERN,
Torry Research Station, Aberdeen.

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It has already been demonstrated [*cf.* Gillam *et al.*, 1931; Edisbury *et al.*, 1933] that although most naturally occurring glycerides, whether saturated or unsaturated, or of animal or vegetable origin, are relatively diactinic, the mixed acids derived from them by prolonged saponification often show intense and highly selective absorption in the ultra-violet. This was found to apply in a particularly striking manner to fish-liver oils and their acids. In a series of acids from several species, narrow absorption bands some 10 $m\mu$ wide were repeatedly recorded at or near 270, 282, 290 (inflection), 301·5, 316·5, 332, 348, 360 (inflection), 377 and 401$m\mu$, and less frequently near 234, 259, 422, 445 and 460 $m\mu$. It was shown that (i) the bands owe their origin to unsaturation in the absorbing molecules; (ii) these highly absorbing acids, of which there appear to be at least five, are not present as the corresponding glycerides in the original freshly extracted oils; (iii) the hypothesis that the acids are open chain compounds is not in harmony with the spectrographic evidence; (iv) a change,

unsaturated acids (low absorption) → unsaturated acids (high and selective absorption),

occurs during the saponification treatment; (v) cyclisation to form polycyclic hydroaromatic compounds is the most plausible explanation of the change; (vi) this change can also occur, spontaneously and without hydrolysis, within the more unsaturated glyceride molecules and is considerably accelerated when the oil is dissolved in a hydrocarbon solvent; but it occurs at best extremely slowly and to a limited extent, involving only a minute proportion of the available material, even when the oil is allowed to stand indefinitely. The change is however quite definite, and is easily detectable if a light source giving a continuous spectrum is employed (*e.g.* hydrogen vacuum tube, or high frequency discharge under water).

We have since found, on the other hand, that the selective absorption of the mixed free acids as usually prepared undergoes little if any change with time; thus, a sample of ling-liver oil acids, which had been kept in nitrogen in a sealed tube for 1½ years, showed little difference between the absorption spectra at the beginning and end of that period, apart from a slight decrease in the persistence of the bands, due probably to the gradual development of general absorption. Richer preparations (*vide infra*) are apparently not so stable. It is entirely unknown what causes the initial development of narrow absorption bands in a neutral liver oil, or what inhibits this spontaneous cyclisation process and prevents the production of more than minimum quantities of cyclised material. For the present, therefore, our attention must be confined to the acid products.

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[1] The substance of this paper was read at the 166th meeting of the Biochemical Society (Liverpool, Dec. 15th, 1934).

Dann and Moore [1933] and Dann *et al.* [1935] showed that although, under suitable conditions, saponification of cod-liver oil was practically complete in a few minutes, the acids so produced exhibited only weakly selective absorption, whilst by prolonging the heating with alcoholic potash the intensity and selective nature of the absorption could be greatly enhanced. They confined their preliminary observations to the middle and further ultra-violet regions. We have confirmed their observations and extended them to the near ultra-violet and the edge of the visible region, and we have found, incidentally, that herring-body oil exhibits the effect quite as strikingly as cod-liver oil. This is interesting because in our earlier work we noted that although the development of highly selective absorption by the saponification products was predominantly a characteristic of oils containing vitamin A, no quantitative connection could be traced between this absorption and the vitamin content. The herring-body oil which we examined (kindly supplied by Prof. Channon) was highly coloured, but gave no blue colour with antimony trichloride, and therefore contained no β-vitamin A. Nevertheless, the results of saponification are in no way inferior to those we have already obtained with a number of fish-liver oils and pancreatic and peritoneal fats, all of which contained vitamin A. Little selective absorption was exhibited by the oil itself in the ultra-violet. The acids obtained as a result of saponification for one minute formed a yellow oil, showing clear bands (though of small persistence) at 270 and 283 $m\mu$ and several others, more poorly defined, nearer the visible. Vigorous saponification for some hours destroyed much of the yellow colour and resulted in greatly increased persistence and definition all round, with an increase in intensity in the middle ultra-violet and a decrease in the region 300–400 $m\mu$. Some substance (possibly an acid pigment exhibiting general or weakly selective absorption in this region and giving rise to the yellow colour of the oil and of the mildly saponified product) was largely destroyed during prolonged saponification. Similar remarks apply, *mutatis mutandis*, to the 270 $m\mu$ portions of the curves for the original oil and the one minute saponification product. In any event, the generally increased persistence of the bands again proves that, despite the reduction in overall intensity above 300 $m\mu$, prolonged saponification has resulted in an increased yield of the highly absorbing acids.

In view of the success attained in securing increased yields by prolonged heating with ethyl alcoholic potash, it was decided to try the effect of saponifying cod-liver oil at different temperatures. Methyl, amyl and octyl alcohols were used as solvents, giving under reflux at atmospheric pressure, reaction temperatures of approximately 70°, 135° and 180° respectively, after allowing for the presence of oil *etc.*, in solution. The spectrographic results are summarised in the first part of Table I.

At this juncture it may be well to explain the system of notation we have adopted for this type of investigation. The figures in Table I record both the absorption intensity and the persistence of the various bands, reduced to a common basis of comparison, namely $E^{1\%}_{1\,\text{cm.}}$, which represents the extinction $\left(\log_{10}\frac{\text{incident light}}{\text{emergent light}}\right)$ of a 1 cm. layer of a 1 % solution at the particular wavelength concerned. The upper figure in each pair gives the $E^{1\%}_{1\,\text{cm.}}$ value at the head or maximum of an absorption band, whilst the lower figure shows approximately the persistence of that band, a quantity which is most conveniently

Table I.

Wave-length (mμ) ...	234	245	260	270	282	290	302	317	332	348	360	377	400
Cod-liver oil saponified for: 1 hour in methyl alcohol	17 Tail-off at 220 mμ. No band	— —	2·1 ~	1·85 *0·12*	1·75 *0·15*	0·77 ~	0·57 ~	0·29 *0·02*	0·16 ~	0·08 ~	0·06 ~	0·04 *0·005*	0·02 ~
1 hour in amyl alcohol	180 *70*	— —	150 *20*	185 *55*	150 *25*	60 ~	31 *7*	28 *12*	4·7 ~?	2·7 *0·9*	0·75 ~	0·5 *0·13*	0·12 *0·025*
6 hours in amyl alcohol	300 *90*	— —	240 *10*	285 *55*	245 *20*	150 ~	75 *15*	65 *15*	23 Very shallow	17 *8*	3·3 ~	3 *1*	0·25 ~
1 hour in octyl alcohol	320 *130*	— —	210 *20*	250 *55*	220 *25*	150 ~	95 *25*	87 *24*	47 *6–7*	29 *11*	7·3 ~	5·7 *1·8–2*	0·8 ~
6 hours in octyl alcohol	270 *ca. 150*	— —	125 ~	145 *20*	137 *12*	95 ~	90 *20*	83 *20*	53 *10*	43 *15–17*	14 ~	8·7–9 *3·5*	0·8 ~
Almost pure $C_{20}F_4$ acids, saponified 6 hours in amyl alcoholic KOH	800 *240*	—	670 *ca. 100*	830 *200*	730 *100*	ca. 350 ~	295 *85*	290 *110*	105 *15*	90 *40*	ca. 25 ~	17·5 *8*	ca. 2·5 ~
Fairly pure $C_{22}F_5$ acids, 6 hours amyl alcoholic KOH	920 *280*	— —	800 *ca. 50*	960 *230*	840 *110*	ca. 500 ~	500 *ca. 150*	460 *ca. 150*	220 *50*	200 *100*	45 ~	50 *25*	3·3 ~
Liquid acids, C_{20}	1030 *300*	850 ~	920 *110*	1040 *240*	910 *110*	ca. 600 ~	240 *60*	200 *50*	55 *ca. 3–4*	40 *17*	8 ~	5·2 *1·2*	ca. 1·5 ~
Semi-solid acids, C_{20}	720 *200*	600 ~	700 *ca. 50*	940 *200*	800 *150*	ca. 500 ~	460 *ca. 130*	460 *ca. 150*	170 *30*	155 *90*	ca. 25 ~	20 *8*	1·6 ~
Liquid acids, C_{22}	930 *270*	870 ~	770 *60*	870 *150*	800 *100*	500 ~	400 *125*	400 *150*	150 *ca. 30*	96 *40*	20 ~	18 *7*	ca. 1·5 ~
Solid acids, C_{22}	500 *150*	420 ~	460 *ca. 10*	600 *30*	660 *100*	— —	860 *200*	930 *300*	750 *220*	730 *350*	ca. 220 ~	220 *120*	ca. 15 ~
Acids from methyl alcohol saponification heated 1 hour at 135° in N_2	Tail-off 30	—	—	6·2 *0·3*	6 *0·1*	4·4 ~	3·3 ~	2·7 *0·2*	1·6 ~	—	0·6 ~	0·3 ~	0·15 ~
Dry neutral soaps heated 6 hours at 135° in N_2	Tail-off 300	—	300 *10*	300 *20*	280 *15*				General absorption				
Original cod-liver oil	Tail-off 10·5	—	—	—	276 mμ 0·29 *0·02*	287 mμ 0·29 *0·02*		313 mμ 0·27 ~	332 mμ 0·32 *0·09*		365 mμ 0·16 ~	—	400 mμ 0·035 ~

Note. ~ denotes inflection. Figures represent $E^{1\%}_{1\,\mathrm{cm.}}$ values; intensity above, persistence (approx.) beneath, in italics, taken as difference between max. and nearest related minimum.

taken as the difference between the $E^{1\%}_{1\,\text{cm.}}$ values at the maximum and at the nearest related minimum (*i.e.* $E_{\text{max.}}-E_{\text{min.}}$). Inflections, by this definition, have no persistence. Owing to the unknown extent to which irrelevant absorption of a general (unselective) nature contributes to the observed total absorption curve, it is necessary to take into account persistence as well as gross intensity of absorption in order to form even a rough estimate of the relative quantities of selectively absorbing material present in different samples. In these experiments, many normally well-defined bands are so completely masked by other absorption as to be rendered imperceptible or reduced in some cases to an inflection with a much higher gross $E^{1\%}_{1\,\text{cm.}}$ value than that of the original band, the net intensity of which may remain indeterminate apart from the fact that an extreme upper limit is set by the observed $E^{1\%}_{1\,\text{cm.}}$ value of the inflection.

It will be seen from Table I that saponification at relatively elevated temperatures rapidly results in a surprisingly good yield of the highly absorbing acids and that temperature is an even more effective factor in their formation than time, so far at least as the acids responsible for selective absorption below 300 $m\mu$ are concerned; a rise in reaction temperature from 70° to 135° accounts for a hundredfold increase in the yield of these acids. Further prolonged heating at the same temperature causes a greater percentage increase in the acids absorbing in the region above 300 $m\mu$ than in those below 300 $m\mu$, and an increase in temperature to 180° again favours those over 300 $m\mu$. On still further prolonging the heating at 180°, a marked decrease is observed in the absorption below 300 $m\mu$, with a roughly corresponding increase above 300 $m\mu$. In the present state of knowledge it seems probable that the acids responsible for the latter are of higher molecular weight and either more complex as regards ring structure and/or more unsaturated than the acids with maxima below 300 $m\mu$.

The precise mechanism whereby cyclisation is induced is by no means clear, but it is worth noting that when the acids from the methyl alcohol saponification were heated in nitrogen for an hour at 135°—the amyl alcohol saponification temperature—the principal change was an all-round but not very marked increase in general absorption, reflected in a perceptible deepening in colour, with an increase in selective absorption only in the case of two bands, 270 and 317 $m\mu$.

An attempt was made on similar lines to cyclise the dry neutral soaps. The product showed practically the same intensity of absorption as the corresponding material from amyl alcohol saponification, but such bands as could be detected were of poor persistence, and some were entirely masked by general absorption. During the heating considerable charring occurred, which would account for much of the general absorption. The presence of a solvent evidently prevents this charring, whilst the alkali (possibly assisted in some way by the solvent) apparently plays an important if at present obscure part in the cyclisation process.

As would be expected, the iodine value steadily decreases during the cyclisation process, from 175·0 for the original oil to 137·6, 129·6 and 115·1 for the products of saponification in amyl alcohol (6 hours) and octyl alcohol (1 and 6 hours) respectively. It is interesting that the iodine value remains as high as it does, suggesting that large quantities of aliphatic unsaturated material must still be present, both in the form of unchanged uncyclised acids and also, probably, as long side-chains attached to partially hydrogenated aliphatic nuclei resulting from the cyclisation process. From the absorption data, it seems unlikely that direct polymerisation is responsible for more than a fraction of the observed decrease in iodine value, although the possibility cannot be altogether excluded that subsequent combination of already cyclised molecules may occur and give rise to polycyclic nuclei absorbing in the near ultra-violet and visible regions. This of course is pure conjecture; nevertheless, the hypothesis is attractive and is not inconsistent with the spectrographic evidence.

Most of the constituents of fish-liver oils are obviously not cyclisable to give acids of the type under discussion. Only the highly unsaturated C_{20} and C_{22} ("clupanodonic") acids, with four and five ethenoid linkages, appear to have a sufficiently long and sufficiently unsaturated carbon chain to provide a suitable starting material. Accordingly it was decided to prepare these C_{20} and C_{22} acids in as pure a state as possible and then to cyclise them. 800 g. of cod-liver oil were saponified in the normal manner, the unsaponifiable matter removed and the acids, still largely uncyclised, recovered. These were submitted once to a lithium salt/acetone separation, yielding 140 g. of highly unsaturated acids which were esterified and distilled *in vacuo*. The following fractions were collected:

Fraction	Weight g.	Boiling-point	Saponification equivalent	Iodine value
1	27·5	130–155°	285·5	212·8
2	42·2	155–165°	313·6	322·5
3	21·7	165–180°	328·1	333·0
4	35·7	180–186°	348·5	340·0
Residue	—		Not examined	

Fractions 2 and 4 were respectively fairly pure C_{20} and C_{22} esters, and were separately cyclised for 6 hours with amyl alcoholic potash; octyl alcohol might have increased the yield, but was not used since it is apt to give exceedingly intractable emulsions. On recovering the acids, the iodine values had fallen to 227·1 for the C_{20} and 251·0 for the C_{22} acids. This would represent a loss in each case of between 1 and 2 double bonds. Polymerisation is again not likely to have been extensive, and it is probable that mixtures of monocyclic, dicyclic and polycyclic products with one or more side-chains were formed. The cyclised C_{20} and C_{22} acids gave qualitatively similar absorption spectra (Table I). This may plausibly be taken to indicate that, so far at least as the major light-absorbing constituents are concerned, there is little essential difference in the nuclei derived from the two fractions—possibly the substitution of an ethyl or one or two methyl groups in the C_{22} product for hydrogen atoms in the C_{20}. The available evidence is not inconsistent with the view that hydronaphthalene derivatives with fairly long side-chains, and also, probably, polycyclic compounds with a low degree of unsaturation, predominate among the absorbing constituents and are distributed roughly equally between the two fractions. The absorption above 300 $m\mu$ further suggests that the C_{22} material is considerably richer in the more unsaturated polycyclic bodies than the C_{20} acids. These latter polynuclear substances appear, however, to be present in relatively small proportions.

There is abundant evidence that several absorbing entities are responsible for the complex absorption curve of the acids. In view of the occurrence of cyclised products it seemed possible that a repetition of the lithium salt/acetone process might effect some separation of the unchanged acids from the others, or of those less highly cyclised (hydronaphthalenic) from those more so (tricyclic *etc.*). The following fractions were obtained:

	Weight in g.	Iodine value
C_{20} acids (liquid)	14·65	231·8
„ (semi-solid)	11·85	225·7
C_{22} acids (liquid)	21·16	259·9
„ (solid)	1·95	203·2

The absorption spectra of these fractions are also shown in Table I, and it will be seen that a very material increase in the concentration of (presumably) polycyclic acids was achieved in the solid C_{22} fraction. Otherwise the degree of separation attained, while by no means insignificant, was disappointing.

Further separation was attempted on both the C_{20} fractions, but the products showed signs of decomposition and polymerisation. For example, although the products, initially clear liquids soluble in light petroleum, were kept under nitrogen, many became gummy and insoluble in the course of a week. Details of this fractionation are given in Tables II *a* and II *b*, to illustrate the type of

Table II *a*. *Attempted separation of C_{20} acids.*

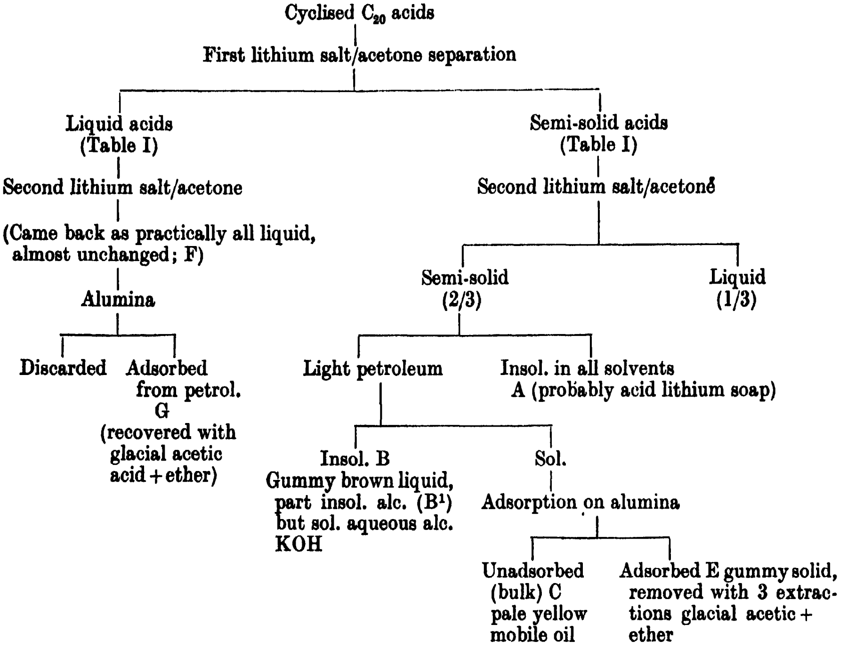
Cyclised C_{20} acids
First lithium salt/acetone separation
Liquid acids (Table I)
Second lithium salt/acetone
(Came back as practically all liquid, almost unchanged; F)
Alumina
Discarded
Adsorbed from petrol. G (recovered with glacial acetic acid + ether)
Semi-solid acids (Table I)
Second lithium salt/acetoné
Semi-solid (2/3)
Liquid (1/3)
Light petroleum
Insol. in all solvents A (probably acid lithium soap)
Insol. B Gummy brown liquid, part insol. alc. (B¹) but sol. aqueous alc. KOH
Sol.
Adsorption on alumina
Unadsorbed (bulk) C pale yellow mobile oil
Adsorbed E gummy solid, removed with 3 extractions glacial acetic + ether

Table II *b*. *Absorption maxima of C_{20} fractions*—cf. *Table II* a.

Fraction	234	260	270	282	290	302	317	332	348	360	377 mμ	
B	530 *200–250*	230 *20*	280 *35*	260 *30*	180 ~	150 *10*	140 *35*	65 *1–2*	43 *13*	20 ~	13 *2·5*	Intensity and persistence in $E^{1\%}_{1\,\text{cm.}}$
B¹	Tail-off 300		Weakly selective $E = 120$ at *ca.* 270 and 285 mμ			(~ 320 mμ? 35)					14 ~	
C	570 *ca. 100*	630 *80*	730 *150*	660 *100*	400 ~	240 *80*	220 *90*	65 *7–8*	50 *25–28*	6 ~	4 *1·5*	
D	570 *200*	500 *ca. 30*	630 *130*	570 *60*	240 ~	210 *60*	180 *50*	65 *ca. 3*	45 *20*	9 ~	4·5 ~	
E	Tail-off 460		Wide ~ 267 mμ 130		Wide ~ 310 mμ 60		(~ also 320 mμ? 33)					
F	800 *ca. 400*	700 *ca. 20*	800 *150*	720 *ca. 70*	240 ~	150 *55*	140 *70*	30 *ca. 10*	22 *10*	4·5 ~	3 *1*	
G	?	300 *10*	320 *20*	300 *10*	200 ~	95 *5*	70 *6*	37 Very low	20	— —	? 7 ~	

Note. Fraction F also exhibited an ill-defined inflection at 249 mμ, intensity $E^{1\%}_{1\,\text{cm.}} = 700$.

behaviour encountered. Fraction B, for example, would be expected to be richer in the highly absorbing acids than fraction C. This may possibly have been the case initially, but by the time (2 or 3 days) that it could be examined spectrographically it had deteriorated markedly and contained a number of flexible tubular bodies, not unlike small worms in appearance and insoluble in alcohol (B^1). These, like the other fractions, refused to crystallise, remaining flexible even at $-20°$. Little apparently is to be gained by repeating the lithium process on the C_{20} acids, and there was insufficient of the C_{22} solid acids available to render an attempt on these profitable. The C_{22} liquid acids polymerised like the C_{20} material.

Owing partly to this instability and partly to a desire to prepare solid derivatives, it was decided to brominate samples of the cyclised acids. In sharp contrast to the uncyclised acids, no precipitate was obtained on brominating the cyclised product in ethereal solution. The bromination was therefore carried out in light petroleum. The C_{20} and C_{22} acids were not in this case freed from monoethylenic esters. 76 g. of cyclised C_{20} acids, and 80 g. of cyclised C_{22} acids, were brominated. In each case, dark green sticky precipitates were obtained, and all attempts at crystallisation failed. A considerable amount of material remained in solution in the light petroleum and was recovered, 90 g. from the C_{20} and 53 g. from the C_{22} acids. Both these petroleum-soluble fractions had a strong camphor-like odour. On debromination with zinc and alcoholic hydrogen chloride, they yielded respectively 25 g. (iodine value 122·5) and 12·5 g. of ethyl esters. The C_{20} insoluble bromides weighed 111 g. and on debromination gave 43·3 g. of ethyl esters (iodine value 171) with an aromatic odour: those from the C_{22} acids resulted in a black tarry product which was not examined. The iodine values mentioned indicate respectively a mean unsaturation of two double bonds and a mixture of mono- and di-ethylenic substances, but spectrographic examination of all the available material showed that very little separation of the selectively absorbing acids had been effected, and that a considerable amount of decomposition had occurred. Bromination as a means of attacking the problem was accordingly abandoned, and other methods of preparing solid derivatives are being attempted.

With a view to confirming the presence of polynuclear ring systems in the structure of the selectively absorbing acids, attempts have been made to identify some of the products of selenium dehydrogenation (Diels's method). The acids from 1 kg. of cod-liver oil were subjected to a lithium salt/acetone separation, and 164 g. of crude highly unsaturated acids obtained. These were converted into ethyl esters and distilled, and distillation was stopped when the esters below C_{20} had come over. The residue (C_{20} and C_{22} esters) was cyclised by saponification for 6 hours in amyl alcohol; the mixture of C_{20} and C_{22} was used in order to provide an adequate quantity of representative starting material. The cyclised product weighed 109 g. During dehydrogenation there was considerable evolution of volatile oily matter (215–250°), presumably alkyl selenides resulting from removal of side-chains from the nuclei. The action then apparently ceased until the temperature was raised to 290–300°, when evolution of hydrogen selenide commenced. The product after heating for 26 hours was taken up with ether, excess selenium filtered off and the filtrate shaken with sodium carbonate to removed unchanged acid. A considerable amount of colour was removed. After washing thoroughly and removing the solvent, a dark brown tarry liquid remained, weighing 78 g. and having an iodine value of 53·8. This was extracted three times with hot alcohol, giving a brown solution with a strong green fluorescence. No solid matter could be recovered from the alcoholic extract, and

Table III *a*. *Distillation of selenium-dehydrogenation product.*

Fractions	B.P. at 2 mm.	Iodine value	Properties
1	60–100°	14·2	Mobile; very pale yellow colour; little or no fluorescence
2	100–130°	17·5	Mobile; pale yellow; slight fluorescence
3	130–160°	23·6	Oily; yellow; violet fluorescence
4	160–200°	42·2	Oily; brown-yellow; violet fluorescence
Residue	—	39·2	Very viscous; brown; green fluorescence
*1 *a*	58– 80°	—	Mobile; almost colourless; no fluorescence

* A further (small-scale) distillation carried out in consequence of the results obtained on fractions 1 and 2.

Table III *b*. *Selenium dehydrogenation.*

n = narrow bands, *f* = fine bands, *a* = aromatic bands.

Crude tar		~ 379* 4·5	~ 343? 11	—	—	—	—	—	—	—	—	—	—	—	*n* 256 110 shallow	
Alc. extract		~ 375 15	~ 347 25	*n* 327 38	—	—	—	—	—	—	—	—	—	*n* 261 230	—	—
					Naphthalenic			*a*								
Fraction 1 *a*		—	—	—	324 1·4	319 2·5 *0·8*	314·5 2·6	311 3	305 5	293·5 22	288 30	283 37	279 50	273·5 52	269 55	*f* 254? 55
										Bands quite distinct, but of low persistence						
		f			Naphthalenic			*a*								
1		377 0·15 *0·05*	359 0·19 *0·025*	*n* ~ or ~ 341 0·25	324 1·1	319 2·2	314 ←	311 (307) 3	304·5 4 →	294 ← 20	289 →	283·5 ←	279 40	274 → ←	270 50	264·5 →
		f			Naphthalenic											
2		377 0·6 *0·25*	359 0·7 *0·15*	*n* 340 1 *0·05*	324 3·3 *0·5*	319 3·5 *0·5*	314·5 4·5	310 6	~ 307 8	~ 295 25	—	—	—	—	*n* 269 55 Poor	*n* 253 70 Good
3	~ 386 0·75	*n* 380 1·2	~ 377 1·15	*n* 360 1·5	~ 349 2·5	*f* 343·5 3·1	*f* 337 3·3	*a* 329 7	*a* 324 7	—	—	—	—	—	~ 267 80	*n* 255·5 115
4	~ 391 2·7	*f* 382 2·9 *0·1*	—	—	~ 350 17	*f* 343–5 20 *2*	—	*n* 329 28 *2*	—	—	—	—	~ 277 180	—	*n* 257·5 290	Tail-off *ca.* 800
Residue		~ 385 25	—	—	—	~ 346? 50	—	—	—	—	—	—	~ 275 370 Shallow	—	—	—

* Possibly incipient 390 $m\mu$ max.

Note. Wave-lengths are given in $m\mu$, with intensity (and, in some cases, persistence), expressed as $E^{1\%}_{1\,\text{cm}}$, beneath.

the solvent was removed, leaving an oily brown liquid. The material insoluble in hot alcohol was a black tarry solid which was not further examined.

Attempts to prepare insoluble picrates from the alcohol-soluble material failed. The picric acid, however, evidently reacted with some constituent of the material, for the green fluorescence disappeared and a red colour developed. On removal of the picric acid with ammonia the original colour and fluorescence were restored. The material was next distilled at 2 mm. pressure and the fractions shown in Table III *a* collected. It seems probable that the true fluorescence of the residue is violet, the green effect arising from a combination of this violet with the natural brown colour of the residue.

All the fractions were examined spectrographically, with the results shown in Table III *b*. An attempt has been made here to classify the types of absorption bands encountered in these fractions—the "narrow" carotenoid type, some 10–20 $m\mu$ wide (*n* in Table), the "fine" band about 5–10 $m\mu$ wide, as exhibited by anthracene and phenanthrene (*f* in Table), and the "aromatic" type, 2–5 $m\mu$ wide (*a* in Table), characteristic of naphthalenic hydrocarbons.

The characteristic absorption maxima of some naphthalenic hydrocarbons are given in Table IV. The similarity between the wave-lengths of the various materials is noteworthy, and whilst amply confirming the presence of a bicyclic aromatic ring system among the dehydrogenation products, emphasises the difficulty of specifically identifying the compound(s) responsible for the absorption. From available data on the relative intensities of absorption at the various maxima, it seems highly probable that a mixture of dimethylnaphthalenes predominates amongst the selectively absorbing products (though present only to the extent of some 5 %), but that naphthalene itself and several of its other derivatives are also likely to be present, together with upwards of 90 % extraneous material exhibiting general absorption. From the well-known inefficiency of the selenium dehydrogenation process (order of yield 3–10 %) the proportion of hydroaromatic bodies in the material before dehydrogenation must have been considerable, though it is conceivable that cyclisation might

Table IV. *Hexane solutions of naphthalene derivatives* (cf. *Table III* b).

Naphth.	1-Me	2-Me	2:6-diMe	1-Me:6-Et	1-Me:7-Et	Δ_2-diH
—	—	~ 325·8	—	—	—	—
320·5	322·2	—	322·8	322	321·5	324·5
	318·7	319	316·2	—	317·2	320·7
~ 314·8	314	314		314·9	315	~ 315
311	—	310·8	310·1	—	—	311
306·8	307	—	—	307	307	306·5
304·5	—	304	—	—	—	303·7
302	302·8	—	303·1	—	—	301
299·8	299	—	—	—	—	—
297·5	—	297·8	296·3	—	—	297
—	293·2	294	—	291	291·8	~ 293
286·5	289	287	—	—	—	287
283·5	281	—	—	280·8	281·8	—
275·5	—	275·1	—	273·5	274	274
272·2	—	—	272 (broad)	—	—	—
—	270·5	—	—	—	—	—
265·5	—	266	—	—	—	267
—	—	—	—	—	—	~ 262
256·5	—	—	—	—	—	—

also have occurred during the early stages of the dehydrogenation process, which appeared to have two well-defined phases.

In any event, however, the presence of aromatic compounds demonstrates that the ability to cyclise is an inherent property of the more unsaturated higher acids. The product is too complex to be worked up by the usual means of picrate or styphnate preparation, owing probably to the presence of partially dehydrogenated products and decomposition products, and also to the presence of several different nuclei in the starting material. Fraction 1, for example, probably contains something of the order of 5 % of mixed dimethylnaphthalenes.

The absence of selective absorption in the region 295–303 $m\mu$ is explicable on the grounds that the absorption curve is here relatively steep, the bands overlapping and masking each other. The bands in the region 330–400 $m\mu$ almost certainly owe their origin to more complex polynuclear systems of the anthracene, phenanthrene, or some higher type. Here again it is at present impossible to identify the substances responsible, for much work remains to be done on such compounds and their derivatives, and the bands here observed do not exactly correspond in position with those given by any polynuclear material for which spectrographic data are at present available. The absorption spectra of aromatic polynuclear materials present, however, a characteristic and unmistakable appearance on the photographic plate, and while this generic similarity increases the difficulty of specific identification of the components of a mixture, it leaves little doubt concerning the type of compound responsible for the absorption.

SUMMARY.

It was shown by earlier work that, although most natural glycerides are relatively diactinic, the acids prepared from them by saponification often show intense, highly selective absorption, particularly in the case of fish oils. Attempts have now been made to concentrate and isolate the absorbing acids and to elucidate their structure. The attempts have been only partially successful, but although the acids have not yet been isolated in a pure state, they have been obtained in much higher concentration than hitherto by prolonged saponification at fairly high temperatures. Thus, saponification for one hour at 135° increased the yield a hundredfold, compared with similar treatment at 70°. Of the methods of separation attempted, the lithium salt/acetone process has given the best results. The solid C_{22} acids from this process represent the best source at present known of the acids absorbing near 350 $m\mu$, and the liquid C_{20} acids of those absorbing near 270 $m\mu$, the concentrations achieved over the original mixed acids (70° saponification) being of the order 10,000 to 1 and 500 to 1 respectively. Cyclisation of the natural C_{20} and C_{22} polyethylenic acids to form hydroaromatic polycyclic derivatives is the most plausible explanation of the changes in absorption, and now receives further support from (1) a marked decrease in iodine values with increasing absorption, and (2) the identification of definite aromatic nuclei, mainly naphthalenic, but some undoubtedly more complex, among the products of selenium dehydrogenation.

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REFERENCES.

Dann and Moore (1933). *Biochem. J.* **27**, 1166.

—— Booth, Golding and Kon (1935). *Biochem. J.* **29**, 138.

Edisbury, Morton and Lovern (1933). *Biochem. J.* **27**, 1451.

Gillam, Heilbron, Hilditch and Morton (1931). *Biochem. J.* **25**, 30.

CIX. THE NON-PROTEIN NITROGEN OF PULSES.

By KAMALA BHAGVAT and MOTNAHALLI SREENIVASAYA.

From the Department of Biochemistry, Indian Institute of Science, Bangalore.

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The saline extract obtained in the preparation of globulins and albumins from pulses contains a non-protein nitrogenous fraction, which is dialysable, not coagulated by heat and not precipitated by saturation with salts or even by reagents such as trichloroacetic acid. Work on foodstuffs relates chiefly to the predominant protein characterising the seed and in the case of pulses the well defined and easily characterisable globulins have received the greatest amount of attention. The methods usually employed for their isolation render recovery of the non-protein nitrogenous bodies extremely difficult and cumbersome. Further, at the moment, there are no systematic methods for investigating a mixture of the complexity represented by this fraction. It is not surprising therefore that this valuable fraction has not hitherto received adequate attention.

It has been suggested that this non-protein fraction arises during the course of the preparation of proteins and that it is not originally present as such in the seed; but experiments have shown that it persists under carefully controlled conditions which preclude its enzymic formation. Whatever be the mechanism of its origin, it has not been generally taken into account in any of the investigations carried out on pulses.

Blish [1918] has investigated the non-protein nitrogen of wheat flour and indicated the probable presence of a non-protein nitrogenous substance not precipitated by copper hydroxide, non-amino and non-peptide in character. Ranga Rao and Sreenivasaya [1933, 1] have shown that the non-protein fractions of the body fluids of the lac insect obtained by (1) salt saturation and (2) coagulation by acetic acid, contain simple polypeptides which have an average complexity (ratio of total to amino-nitrogen) of 4·7. They [1933, 2] have further demonstrated the presence of free tyrosine which occurs to the extent of about 2·5 % of the non-protein fraction. Sreenivasan and Subrahmanyan [1934] have drawn attention to the presence of non-coagulable forms of nitrogen in the saline extract of leguminous seeds which may have great nutritive value.

In view of their simpler structure these nitrogenous compounds should be among the most easily digestible and assimilable; they may help the peptisation of the associated proteins and influence their enzymic digestion by providing the necessary activators and further they may supplement the nutritional deficiencies of the associated proteins and enhance their biological value. It is therefore obvious that this non-protein fraction merits a detailed investigation from all points of view and the present study relates to a determination of the percentage and average complexity of the non-protein nitrogen lost during the preparation of proteins by various methods.

EXPERIMENTAL.

Nine of the well-known Indian pulses, after air-drying, were powdered to pass through a 40-mesh sieve; with the exception of *Cajanus indicus*, *Lens esculenta*, *Cicer arietinum* and *Phaseolus mungo* which could be easily freed from husks, the pulses were powdered along with the husk. The meal (100 g.) was extracted for 24 hours at 0° with 100 ml. of 5 % NaCl by shaking the mixture at frequent intervals. On filtering the mixture a clear solution was obtained. The entire operation was carried out in a refrigerator maintained at 0° and in presence of toluene.

Total nitrogen was determined by the Kjeldahl method and amino-nitrogen by the method of Van Slyke in all the solutions examined. Ultrafiltration was carried out through collodion membranes under a pressure of 50 kg. per cm.^{2} employing 50 ml. of the saline extract, which was kept electromagnetically stirred during the filtration. This facilitated rapid filtration and prevented clogging of the pores of the membrane.

The trichloroacetic acid filtrate was obtained by treating 20 ml. of the saline extract with 5 ml. of 20 % trichloroacetic acid. The precipitate was filtered off, washed with 2 % trichloroacetic acid and the filtrate made up to 50 ml. Aliquots of 20 and 10 ml. were used for the total and amino-nitrogen respectively.

20 ml. of the saline extract were kept in a steam steriliser for 1 hour, the coagulated protein was filtered off, washed with 5 % NaCl and the filtrate made up to 50 ml. 20 and 10 ml. were used for the total and amino-nitrogen respectively.

Dialysis was carried out in collodion bags (prepared from a 6 % solution of pyroxylin (B.D.H.) in ether-alcohol mixture) against distilled water until the dialysate was free from chloride. The contents of the bag along with the precipitated protein were transferred to a beaker and treated with a sufficient quantity of sodium chloride to redissolve the precipitate and the volume was made up to 150 ml. with 5 % NaCl. An aliquot of 20 ml. was used for total nitrogen. The difference between this value and the total nitrogen of the crude extract gave the value for the diffusible non-protein nitrogen.

Ammonium sulphate was employed for the salt saturation experiments, since it is commonly used in all preparative work. The precipitate thus obtained was centrifuged, washed with a saturated solution of ammonium sulphate, redissolved in water and reprecipitated with 10 ml. of 20 % trichloroacetic acid. The precipitate was filtered off, washed with 2 % trichloroacetic acid until free from ammonia and used for determination of the total nitrogen. The difference

Table I.

	Percentage of nitrogen lost during the isolation of proteins by				
	Ultra-filtration	Trichloro-acetic acid pptn.	Heat coagulation	Dialysis	Saturation with $(NH_4)_2SO_4$
Cicer arietinum	12·1	19·1	—	19·6	23·4
P. mungo	9·3	24·7	—	—	24·2
P. radiatus	8·5	7·1	12·7	—	—
Vigna catiang	11·9	14·5	23·6	25·4	39·8
Dolichos Lablab	16·0	14·9	36·0	4·6	26·1
Cajanus indicus	14·3	23·5	45·6	25·7	23·6
Lens esculenta	26·1	29·3	46·2	52·1	43·6
P. aconitifolius	27·6	29·5	40·5	55·3	43·3
Dolichos biflorus	20·7	29·2	55·1	55·2	40·7

between this value and the total nitrogen of the saline extract represents the non-protein fraction not usually investigated.

Table I gives the percentage of nitrogen lost during the isolation of proteins by several methods adopted, whilst Table II gives the average complexity of the nitrogenous bodies in the crude saline extract and in the several non-protein fractions.

Table II. *Average complexity of the nitrogenous bodies in the crude saline extract and in the non-protein fraction.*

	Crude extract	Ultrafiltrate	$CCl_3.COOH$ pptn.	Heat coagulation
Cicer arietinum	10·6	3·8	3·5	6·3
P. mungo	13·6	2·5	3·7	7·0
P. radiatus	15·9	1·8	2·8	4·5
Vigna catiang	10·4	2·3	2·1	3·1
Dolichos Lablab	11·2	2·9	2·6	6·8
Cajanus indicus	6·4	2·0	2·0	4·0
Lens esculenta	7·1	2·5	2·9	4·8
P. aconitifolius	7·0	2·0	2·7	2·8
Dolichos biflorus	7·4	2·6	2·6	4·4

DISCUSSION.

The non-protein nitrogen has been determined by five different methods each of them representing a well-defined process conventionally adopted in the study of proteins. The quantity thus lost varies from 10 to 55 % of the nitrogen extractable by saline solution, depending upon (1) the nature of the pulse under investigation and (2) the method adopted for the estimation of the non-protein fraction. Trichloroacetic acid is usually employed as a precipitant for proteins in various physiological fluids. Dialysis is a recognised method for the isolation of the globulins which are thrown out of solution as the dialysis proceeds, while saturation with ammonium sulphate offers a convenient method for the precipitation and purification of globulins and albumins. Ultrafiltration was adopted as a method of isolating the non-protein nitrogen of the saline extracts, since it offers possibilities of fractionating the nitrogenous bodies without contaminating extracts with acids and salts difficult to eliminate at a later stage. In the present study however only one grade of membrane has been used for all the pulses investigated.

Heat brings about coagulation not only of the globulins but also of the albumins and practically the same result is achieved by saturation with ammonium sulphate but with this difference, that the proteins are not denatured. There should therefore be fair agreement between the two sets of values; such agreement does indeed exist in the cases of the last three pulses in Table II. The higher the average complexity of the nitrogenous bodies in the crude extract, the less will be the nitrogen lost in the filtrate after heat coagulation. *P. radiatus*, for example, whose crude saline extract has the highest average complexity in the series (15·9), suffers the least loss of nitrogen not only during heat coagulation but also during ultrafiltration and precipitation with trichloroacetic acid. Similarly it will be observed that the last four pulses in Tables I and II, whose nitrogenous bodies in the saline extract possess lower complexities (6·4–7·4), yield high percentages of the non-protein fraction on heat coagulation and also on ultrafiltration and precipitation with trichloroacetic acid.

It will be seen that there is general agreement between ultrafiltration and trichloroacetic acid precipitation except in the case of *P. mungo*, which indicates that the membrane used for ultrafiltration is impermeable to all that the trichloroacetic acid is able to precipitate. This is born out by the fact that none of the ultrafiltrates yields any precipitate with trichloroacetic acid.

The average complexities of the "non-protein" fractions obtained by ultrafiltration and precipitation with trichloroacetic acid, in the case of the pulses examined, are always lower than the corresponding figure for the fraction obtained by heat coagulation, whose uniformly higher complexity indicates the presence or peptones and higher polypeptides in the filtrate. Further confirmation of this fact is obtained by the observation that the filtrates after heat coagulation give a further precipitate with trichloroacetic acid. Particular interest attaches to the heat-coagulated fraction since heat coagulation represents the nearest approach to the conditions of cooking and corresponds to the portion generally administered to invalids and children. It is interesting to observe that the average complexities of the fractions in general run parallel with the recognised ease of their digestibilities. The non-protein fraction of *P. aconitifolius*, for instance, has the lowest ratio of 2·8 in the heat coagulation series, a fact which is in harmony with the reputation which this pulse enjoys as a very easily digestible and assimilable source of nitrogen during convalescence. This is apparently contradictory to the findings of Niyogi *et al.* [1932] that the protein of *P. aconitifolius* is poor both as regards its digestibility and biological value. It only suggests the supplemental value of the non-protein fraction and emphasises its importance in influencing the digestibility and biological value of the protein.

The saline extracts, the ultrafiltrates and the heat-coagulated filtrates were examined to see if they contained any of the essential amino-acids in a free condition. An active preparation of tyrosinase from *Dolichos Lablab*, was employed to test for tyrosine and related compounds like 3:4-dihydroxyphenylalanine. In view of the high concentration of chlorides and carbohydrates, tryptophan could not be tested for with glyoxylic acid. Results of the tyrosine test, given in Table III, indicate that only two of the pulses examined, *P. aconitifolius* and *P. radiatus*, show the presence of tyrosine definitely, the former containing a much higher concentration of the amino-acid, in all the three different filtrates tested. In the case of *Dolichos*, tyrosine could not be detected in the heat-coagulated filtrate, while its presence was unmistakable in the crude extract and in the ultrafiltrate. This observation together with the fact that the *Dolichos* extract gradually darkens on keeping, points to the conclusion that tyrosine is gradually liberated from the proteins through the action of the associated protease which becomes inactivated during the process of heat coagulation.

Table III.

	Saline extract	Ultrafiltrate	Heat-coagulated filtrate
P. aconitifolius	+ + +	+ + +	+ + +
P. radiatus	+	+	+
D. Lablab	+	+	–

SUMMARY.

The saline extracts obtained during the preparation of globulins and albumins from pulses, contain a non-protein nitrogenous fraction, which occurs to the extent of 10–55 % of the total nitrogen depending upon the nature of the pulse and the method of determination.

This non-protein fraction has been shown to contain the simpler, easily digestible and assimilable peptides which have an average complexity varying from 1·8 to 3·8. In some cases the existence of free tyrosine or simple derivatives thereof has been demonstrated.

Attention has been drawn to the fact that heat coagulation constitutes the nearest approach to the conditions of cooking and the fraction thus obtained represents the portion usually administered to children and invalids.

Our thanks are due to Dr V. Subrahmanyan for his kind interest in the work.

REFERENCES.

Blish (1918). *J. Biol. Chem.* **33**, 551.
Niyogi, Narayana and Desai (1932). *Ind. J. Med. Res.* **19**, 859.
Ranga Rao and Sreenivasaya (1933, 1). *J. Ind. Inst. Sci.* **16** A, 76.
—— —— (1933, 2). *Current Sci.* **1**, 378.
Sreenivasan and Subrahmanyan (1934). *J. Ind. Inst. Sci.* **17** A, 49.

CX. THE EFFECT OF HYPNOTICS ON GLUCOSE TOLERANCE.

BY HENRY TOD[1].

From the Royal Edinburgh Mental Hospital.

(*Received February 28th, 1935.*)

IN the course of an investigation into the glucose tolerance of mental patients, four extremely abnormal curves were obtained. In these there was little or no initial rise in blood sugar after the ingestion of 50 g. glucose and the blood sugar fell to low levels. The only abnormality found in the circumstances of these curves was that in each case the patient received a dose of a hypnotic previous to the ingestion of the glucose. It was found, furthermore, that in each case the drug had been administered at such a time previous to the administration of the glucose that the patient was just coming under the influence of the drug when the test was commenced. Thereafter, hypnotics were not given before carrying out the tests, but time did not permit of a more detailed investigation of the problem.

Subsequently, this question has been examined more closely. A search of the literature shows that while numerous workers have investigated the effects of hypnotics on the fasting blood sugar, the only workers who have investigated their effects on glucose tolerance tests are Högler and Zell [1933] who state that they find that neither veronal nor chloral has any marked effect on alimentary hyperglycaemia in rabbits. An examination of their results shows, however, that eight out of nine experiments carried out with these drugs show the effect described below.

In the following research a glucose tolerance test was carried out on the fasting patient and within a few days, often the next day, the test was repeated with the patient fasting and under the influence of a drug. In a number of cases a repetition of the "normal" curve was carried out, and in each case the results were comparable.

This work has been carried out on two classes of patients: (*a*) cases showing marked lack of emotion and apathetic behaviour. These cases showed also considerable intellectual deterioration and for descriptive purposes are referred to as cases of "dementia" in this paper in contrast to (*b*) cases which showed the typical emotion consonant with their type of psychosis and had fairly well-maintained personalities. It is noticeable, however, that the phenomenon is manifested independently of the type of disorder.

The drugs used were:

(1) Nembutal, gr. iii;
(2) Veronal, gr. vii or x;
(3) Chloral, gr. xx;
(4) Morphine sulphate, gr. $\frac{1}{4}$ or $\frac{1}{6}$;

i.e. the ordinary clinical doses used in hospital practice.

No very profound variations were found in the fasting levels whether the patient was under the influence of the drug or not, and for clarity the fasting levels of the two curves have been made equal. Blood sugar estimations were carried out on oxalated-fluorided blood by the Hagedorn-Jensen technique.

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[1] Walter Smith Kay Research Fellow in Psychiatry, University of Edinburgh.

The tables show the fasting level, the maximum level attained and the 2 hour level or the minimum if that is attained before 2 hours. In addition, the hyperglycaemic index (H.I.) and the hyperglycaemic area (H.A.) are shown for, in each case, first the normal curve and, second, the curve after the drug has been administered. In each case the age, sex and diagnosis for the patient are given.

It will be seen that in most cases following the administration of the drug there is a fall in the H.I., excepting where the "normal" was zero. In each case, however, the hyperglycaemic area shows a fall. This is expressed as a percentage decrease, calculated from the formula:

$$\%\ \text{decrease} = \frac{(\text{Area}_{(\text{no drug})} - \text{Area}_{(\text{drug})}) \times 100}{\text{Area}_{(\text{no drug})}}.$$

The hyperglycaemic area was found by actual measurement by counting squares when the curves were plotted on 1/10 in. graph paper. The hyperglycaemic index (H.I.) is that of McCowan and Quastel [1931]:

$$\text{H.I.} = \frac{(2\ \text{hour level} - \text{fasting level}) \times 100}{(\text{maximum} - \text{fasting level})}.$$

Where a minimum is reached before 2 hours this value is used. In addition to the H.I. and decrease in H.A. the change in the curves may be seen from the maxima and minima in the tables. In the following tables the curves marked (*a*) are the "normal" curves and those marked (*b*) are those obtained when the patient was under the influence of the drug.

Table I.

A. *Nembutal.*

Case	Blood sugar mg./100 ml.			H.I.	H.A.	% decrease in H.A.	Sex	Age	Diagnosis
	Fasting	Max.	Min.						
i (*a*)	70	80	72	20	9	—	M	45	Mania
(*b*)	70	(40)	55	—	0	100			
ii (*a*)	80	105	92	50	32	—	M	56	Melancholia
(*b*)	80	100	75	0	18	56			
iii (*a*)	75	115	60	0	24	—	M	66	Inv. Mel.
(*b*)	75	95	45	0	17	29			
iv (*a*)	80	150	135	80	152	—	M	57	Acute Dep.
(*b*)	80	130	65	0	52	66			
v (*a*)	120	240	210	75	280	—	F	64	Dementia
(*b*)	120	220	170	50	194	31			
vi (*a*)	70	120	80	20	64	—	F	68	Dementia
(*b*)	70	95	70	0	32	50			
vii (*a*)	75	151	120	60	130	—	M	76	Dementia
(*b*)	75	120	120	100	63	49			

Inv. Mel. = Involutional melancholia, Dep. = Depression.

In case (i) the hyperglycaemic rise is entirely abolished, the blood sugar content falling to a minimum at 1 hour and then rising slowly. Case (i) was very excited at the time of the test. The other cases show the typical lowering of the curve. Case (vii), however, shows a rise in H.I., although the H.A. shows a 49 % decrease.

Owing to lack of space, it is not possible to show the individual pairs of graphs and accordingly the following method has been used for a graphic representation. At each time period, *i.e.* ½, 1, 1½, 2 and 2½ hours after the ingestion of glucose the blood sugar level for the curve on the drug was subtracted from the blood sugar level on the control curve, and the difference was termed the "depression" for that time. The means of these "depressions" for each time and for each drug were determined and are shown in Table V together with the maximum and minimum depressions.

Table II.

B. *Veronal.*

	Blood sugar mg./100 ml.								
Case	Fasting	Max.	Min.	H.I.	H.A.	% decrease in H.A.	Sex	Age	Diagnosis
viii (*a*)	75	100	70	0	12	—	F	37	Ag. Depr.
(*b*)	75	75	55	—	0	100			
ix (*a*)	80	140	90	17	57	—	F	52	Ag. Depr.
(*b*)	80	105	80	0	23	40			
x (*a*)	95	127	90	0	25	—	M	62	Ag. Depr.
(*b*)	95	120	90	0	11	44			
xi (*a*)	80	107	85	20	33	—	F	49	Inv. Mel.
(*b*)	80	102	70	0	17	48			
xii (*a*)	80	140	65	0	68	—	F	?	Mania
(*b*)	80	125	35	0	38	44			
xiii (*a*)	75	140	90	23	70	—	F	59	Dementia
(*b*)	75	105	70	0	30	57			
xiv (*a*)	60	140	130	88	161	—	F	49	Dementia
(*b*)	60	125	95	54	102	37			

Ag. Depr. = Agitated depression.

In case (viii) also, the hyperglycaemic rise is abolished; a small transitory rise may however have been missed between the fasting sample and the sample drawn 30 minutes after the ingestion of the glucose. No case showed a rise in H.I. and the other cases show the typical increase of tolerance.

Table III.

C. *Chloral.*

	Blood sugar mg./100 ml.								
Case	Fasting	Max.	Min.	H.I.	H.A.	% decrease in H.A.	Sex	Age	Diagnosis
xv (*a*)	80	175	110	32	130	—	F	54	Inv. Mel.
(*b*)	80	150	105	36	94	28			
xvi (*a*)	80	113	100	61	63	—	M	24	Melancholia
(*b*)	80	103	75	0	26	59			
xvii (*a*)	90	118	80	0	26	—	M	40	Melancholia
(*b*)	90	98	70	0	8	69			
xviii (*a*)	80	135	95	27	61	—	F	70	Dementia
(*b*)	80	107	75	0	18	70			
xix (*a*)	80	115	105	71	67	—	F	28	Dementia
(*b*)	80	100	90	50	35	48			
xx (*a*)	85	130	115	67	75	—	F	63	Dementia
(*b*)	85	125	105	50	57	52			
xxi (*a*)	75	100	85	40	39	—	F	37	Dementia
(*b*)	75	90	70	0	8	79			

No case with chloral showed the abolition of rise in blood sugar. One case (xv) shows a rise in H.I., the other values for this case show depression.

Table IV.

D. *Morphine.*

Case	Blood sugar mg./100 ml.								
	Fasting	Max.	Min.	H.I.	H.A.	% decrease in H.A.	Sex	Age	Diagnosis
xxii (*a*)	90	145	105	27	83	—	M	77	Sen. Hyp.
(*b*)	90	127	113	62	43	48			
xxiii (*a*)	85	169	85	0	78	—	M	41	Schiz.
(*b*)	85	94	80	0	7	89			
xxiv (*a*)	80	142	135	40	125	—	M	77	Sen. Dem.
(*b*)	80	124	118	86	94	25			
xxv (*a*)	80	135	95	27	30	—	F	60	Dementia
(*b*)	80	92	88	66	13	57			
xxvi (*a*)	75	121	100	54	72	—	F	52	Dementia
(*b*)	75	108	68	0	17	76			
xxvii (*a*)	85	97	64	0	9	—	F	37	Dementia
(xxi) (*b*)	85	87	74	0	1	89			
xxviii (*a*)	85	125	105	50	65	—	M	74	Dementia
(*b*)	85	99	99	100	22	66			

Sen. Hyp. = Senile hypochondriasis, Schiz. = Schizophrenia, Sen. Dem. = Senile dementia.

In this series only one case showed a fall in H.I., four showed a rise and two did not change (H.I. = 0). A reason for this will be suggested later. The areas, however, show a marked decrease with the drug.

Table V. *Differences in blood sugar content due to hypnotics.* (mg./100 ml.)

Hours	... ...	½	1	1½	2	2½
Nembutal	Mean	-17	-23	-24	-24	-11
	Max.	-40	-60	-40	-70	-25
	Min.	- 5	+ 5	- 5	0	+25
Veronal	Mean	-20	-16	-21	-14	- 3
	Max.	-45	-25	-40	-35	-25
	Min.	- 5	-10	-10	+ 5	+10
Chloral	Mean	-18	-16	-13	-15	-11
	Max.	-40	-25	-20	-30	-35
	Min.	-10	0	0	- 5	0
Morphine	Mean	-31	-30	-21	- 4	+ 8
	Max.	-50	-55	-35	-25	-10
	Min.	- 5	-15	+ 5	+ 5	+25

A normal blood sugar curve [Stewart and Dunlop, 1932] was taken as a standard, and the mean "depressions" were subtracted from its values for each drug. Figs. 1–4 show the depressed curves so obtained.

From Fig. 4 it will be seen that at 2 hours the depression is small and at 2½ hours an increase is actually found. Further, the maximum of the curve (½ and 1 hour) is heavily depressed (Table V). This accounts for the elevation of the H.I. in so many of the morphine cases.

Seven cases of schizophrenia were used, and an interesting point was shown. In all cases but one (xxiii) the drugs had no clinical action whatever, and in each case the two curves obtained were identical. Case (xv), for some reason had no clinical reaction to the drug in the first instance and two identical curves were obtained. On a subsequent occasion the drug was effective with the results shown above. This fact is particularly clear and seems to indicate that the biochemical manifestations run closely parallel with the clinical effects.

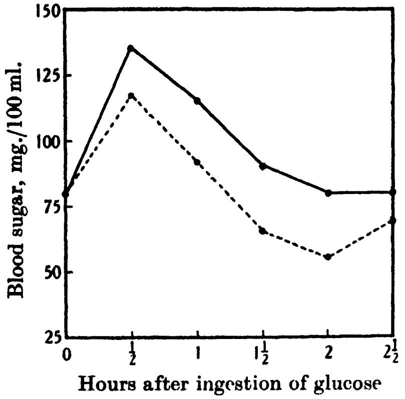

Fig. 1. Nembutal.

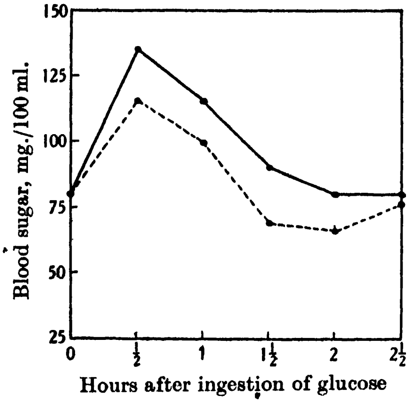

Fig. 2. Veronal.

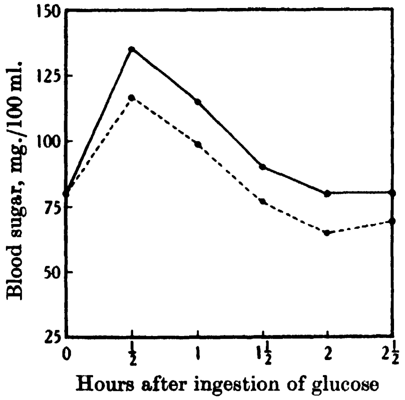

Fig. 3. Chloral.

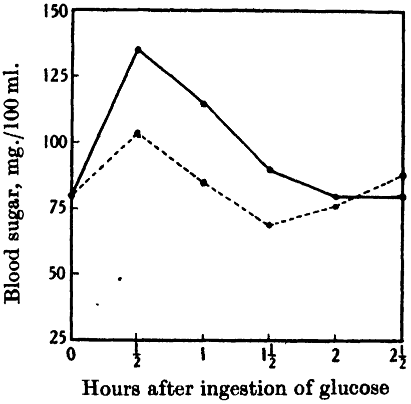

Fig. 4. Morphine.

SUMMARY.

1. It has been shown that hypnotics in clinical doses show a marked effect on the results of the glucose tolerance test.
2. This effect is shown by lowered maxima or shortened hyperglycaemia, or both, with markedly decreased hyperglycaemic area. The effect on the H.I. is less constant.
3. This effect is shown by chloral and morphine and is, therefore, not peculiar to drugs of the barbituric group.

I wish to thank Prof. D. K. Henderson for providing me with facilities to carry out this work, Dr T. A. Munro for his helpful cooperation in handling the case material, and the Medical Research Council for a personal grant enabling me to finish the work.

REFERENCES.

Högler and Zell (1933). *Z. ges. exp. Med.* **86**, 185.
McCowan and Quastel (1931). *J. Ment. Sci.* **77**, 525.
Stewart and Dunlop (1932). Clinical chemistry in practical medicine, p. 78 (Edinburgh).

CXI. THE KINETICS OF ALCOHOLIC FERMENTATION OF SUGARS BY BREWER'S YEAST.

I. EFFECT OF CONCENTRATIONS OF YEAST AND SUGAR.

By REGINALD HAYDN HOPKINS
and RICHARD HENRY ROBERTS.

From the Department of Industrial Fermentation, University of Birmingham.

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The kinetics of alcoholic fermentation of sugars by living yeast was established by the work of Brown [1892] and Slator [1906; 1908]. These workers showed that the rate of fermentation is approximately constant over a wide range of sugar concentration and remains constant during the main part of the fermentation. Brown used a high seeding rate which, in the absence of added nutrients other than the sugar, remained fairly constant during the observations, and measured the rate of fermentation by the rate of disappearance of the sugar. On the other hand Slator used a low seeding rate and measured in a specially devised apparatus the velocity of CO_2 evolution over short periods of time during which no yeast increase could take place. He calculated the value of "n" in the equation

$$\frac{d\,[CO_2]}{dt} = k\,[\text{sugar}]^n,$$

and showed that it was approximately zero between the limits of sugar concentration 0·5 % and 10 %. Slator and Sand [1910] concluded that "...it is unlikely that conditions could be experimentally realised under which diffusion becomes a controlling factor in the rate of fermentation". Their deductions also indicated the importance of uniform shaking or stirring of the fermenting mixture.

Nord and Weichherz [1929] criticised Slator's apparatus and considered that,

(1) it had no adequate stirring device whereby continuity of reaction could be secured;

(2) it exhibited large temperature fluctuations;

(3) readings could not be made soon enough after the commencement of the fermentation.

Using the Iterson-Kluyver apparatus to measure the rate of evolution of carbon dioxide they investigated the effects of varying stirring rates and found that with 21 turns per minute or more the rate of fermentation exhibited three phases:

(1) a fairly rapid rise to a maximum;
(2) a short quasi-stationary period;
(3) a rapid asymptotical decrease to zero.

Phase (2) was absent when no stirring took place. The constancy of fermentation rate over a relatively long period of time, observed by Brown and Slator, was not observed in these experiments.

The method of stirring or agitation is important. Slator shook vigorously by hand at intervals. Nord and Weichherz assumed that all diffusion gradients in the sugar solution are eliminated by stirring at the rate of 21 turns per minute, and attributed the further increase in maximum rate of fermentation consequent upon increased rate of stirring to an apparent increase in the membrane surface due to the motion of the cells relatively to the medium. But in the fermentations performed by them, the yeast rate was so high (2 g. of pressed yeast in 20 ml. total volume) that the cells, if uniformly distributed, would be separated from each other by a distance considerably less than one diameter. It seems probable that diffusion gradients and vortex effects would occur even at higher stirring rates. However, by maintaining a steady stirring rate, comparable results were obtained. Weichherz [1929; 1931] from mathematical considerations concluded, among other things, that proportionality between rate of reaction and dry weight of yeast employed does not necessarily hold. It may not hold in such cases as the above, but it was found by Slator to hold for low concentrations of yeast, and this has been confirmed by us (Exp. 2) over a wide range of yeast concentration.

In further experiments, Nord and Weichherz, using a stirring rate of 100 turns per minute, investigated the effect of glucose concentration on the maximum rate of fermentation and found the latter to increase up to 10 % glucose, whereas Slator had found little deviation over the range 0·5–10 %, and the maximum at 4 %.

A closer examination of the results, however, indicates that they may be interpreted to agree with those of Slator. In each of the fermentations recorded by Nord and Weichherz, a large yeast rate was employed and a large time lag (phase (1)) ensued before the maximum rate was attained. During this lag an appreciable amount of sugar was decomposed, and this amount can be calculated from the graphical data furnished. For example, in the case of 0·5 % initial glucose, the maximum rate was attained after fermentation had decreased the sugar concentration to 0·31 %. The maximum rate may therefore be taken to apply to this concentration and not to 0·5 %. Similar results calculated from the other cases are included in the table below.

Table I.

Initial glucose %	Time taken to attain maximum rate (mins.)	Glucose remaining at time of maximum rate (%)	Rate of fermentation ml. CO_2/min.
0·5	10	0·31	1·40
2·0	30	0·96	2·45
5·0	38	3·48	2·85
10·0	70	6·14	3·05
30·0	82	26·11	2·90

By plotting these maximum fermentation rates against the appropriate concentrations of glucose, a curve is obtained (Fig. 1) which shows close agreement with the corresponding curve of Slator [1906] and, in fact, demonstrates the "approximate independence" of sugar concentration more clearly than Slator's original curve. A deduction in support of this may be made from the equation derived from first principles by Weichherz [1929; 1931] who found that the velocity of fermentation

$$f = \frac{\mu k}{k-\mu} \cdot S_0 \left(e^{-\mu t} - e^{-kt}\right),$$

where S_0 = the initial substrate concentration in the outer medium,
k = the unimolecular constant of the reaction proceeding in the cell,
and $\mu = \frac{F\alpha D}{v\delta}$,

in which F = cell (membrane) surface,
δ = membrane thickness,
αD = diffusion coefficient of the substrate within the membrane (this diffusion coefficient is derived from the free diffusion coefficient (D) in the total system filled with solution, and α the degree of permeability of the membrane),
and v = cell volume.

The above equation can be solved for k and μ if sufficient values of f and t are known. Let us suppose the following values are known:

$$t=1 \quad f=f_1$$
$$t=2 \quad f=f_2$$
$$t=3 \quad f=f_3.$$

By substituting these values in the equation, followed by usual algebraical methods, it can be shown that

$$e^{-\mu}+e^{-k}=f_2/f_1$$
$$e^{-\mu}\times e^{-k}=(f_2/f_1)^2-f_3/f_1,$$

whence we can calculate k, the true unimolecular constant of the reaction proceeding inside the cell. Application of this theorem to the results of Nord and Weichherz revealed that the value of k decreased with increase in initial concentration of glucose. Although the reaction proceeding within the cell is unimolecular, it does not proceed unimolecularly in the presence of any appreciable external sugar concentration since the velocity of reaction, $f=ka$, is maintained by continuous replacement of sugar by diffusion.

It was considered desirable to investigate the following points which arise out of the above discussion;

(1) the effect of stirring or shaking (Exp. 1);

(2) the relationship between concentration of yeast and rate of fermentation (Exp. 2);

(3) the relationship between concentration of sugar and rate of fermentation (Exp. 3).

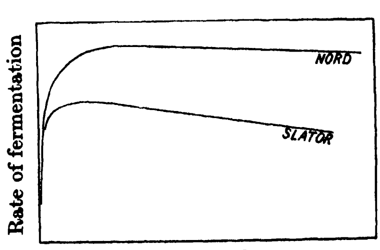

Fig. 1.

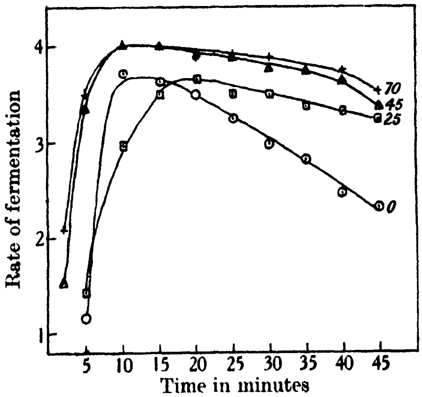

Fig. 1a.

EXPERIMENTAL.

The apparatus used was essentially that described by Slator [1906] in which the fermentation vessel is connected with an exhaust pump and a mercury manometer, change of pressure being observed as fermentation proceeds. Modifications, some of which were designed to overcome the objections advanced by Nord and Weichherz, included a two-way tap connecting to the exhaust pump or the air, and a shaking device for rocking the vessel to and fro and imparting a jerk, the rate of shaking being controlled by a rheostat in conjunction with the motor. Shaking was adopted in preference to stirring. The fermentation vessel, 250 ml. in capacity, was completely immersed in a constant temperature bath and the sugar, in solution (mutarotated), contained in a small beaker supported on a float on the surface of the yeast suspension previously placed in the vessel. Glass beads were present to assist dispersion of the yeast. When the whole system had settled down to the required temperature (30° in most experiments, as also adopted by Slator and Nord and Weichherz) the apparatus was exhausted and the shaking commenced; the beaker discharged its contents into the yeast suspension and readings on the manometer scale were taken from the beginning of the fermentation. These readings were plotted against time in each experiment, the rates of fermentation at selected arbitrary times deduced from the slope of the curve and calculated to the units, mg. of CO_2 per minute, as expressed in the various tables[1]. For this purpose the apparatus was calibrated as follows. 2 ml. of solution of pure sodium carbonate containing 65·8 mg. of CO_2 were placed in a small beaker floating on the surface of 98 ml. of sulphuric acid, of such concentration as to be exactly equivalent, contained in the fermentation vessel of the apparatus. When the system had attained the desired temperature, the pressure was reduced, the reaction flask shaken and the change of pressure on liberation of CO_2 was observed. In the case described, 100 ml. of solution in the flask in liberating 65·8 mg. of CO_2 at 30° brought about an increase of pressure equal to 5·8 cm. of mercury. Similar calibrations were performed for other volumes of liquid in the reaction vessel and for other temperatures as required.

Pressed washed brewer's (top) yeast from the same source was used in all experiments. By using yeast one day old, autofermentation was invariably found to be negligible.

Exp. 1 a. Variation of rate of shaking.

Glucose 0·5 g. (1 %). Yeast, 8 g.
Temp. 35°. Total volume 50 ml.

Table 1 A. *Progressive rates of fermentation expressed as mg. CO_2 per minute.*

Time (mins.) ...	2	5	10	15	20	25	30	35	40	45
No shaking	—	1·17	3·76	3·63	3·50	3·24	2·98	2·72	2·46	2·32
25 shakes per minute	—	1·43	2·98	3·50	3·63	3·50	3·50	3·37	3·32	3·24
45 shakes per minute	1·55	3·37	4·02	4·02	3·94	3·89	3·76	3·76	3·63	3·37
70 shakes per minute	2·09	3·50	4·02	4·02	3·89	3·89	3·89	3·89	3·76	3·50

It can be seen from the graph (Fig. 1*a*) that the rate of shaking is important. The effect of shaking is presumably most marked when high yeast rates are employed, as in this case. However, 70 and 45 shakes per minute gave similar results. It is reasonable to suppose that intermediate rates would do the same, but in all subsequent experiments shaking was maintained at approximately 70 shakes per minute.

The quasi-stationary periods were much longer than the corresponding ones observed by Nord and Weichherz, although the yeast rate was even higher than that employed by them.

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[1] An experimental error of, *e.g.*, 1 mm. in the scale reading, spread over 30 mins. in the graphical procedure outlined above, amounts to less than 0·04 mg. CO_2 per minute.

Exp. 1b. Effect of intermittent shaking.

Parallel fermentations were performed:

(*a*) with continuous shaking at 70 shakes per minute;

(*b*) with alternate periods, without shaking and with vigorous shaking (over 70 per minute);

(*c*) without shaking.

In all cases of intermittent shaking, the shaking periods were continued until the accelerated rate of gas evolution had ceased and the normal rate with shaking was attained.

Glucose 0·5 g. Yeast 5 g.
Temp. 30°. Total volume 50 ml.

The results are shown graphically in Fig. 1 *b*.

Exp. 1c. The Exp. (1 *b*) was repeated for conditions (*a*) and (*b*), but in the case of (*b*) longer time intervals, 14 minutes, were allowed to elapse between the periods of shaking. The object of this was to exaggerate any effect due to intermittent quiescent periods. The concentration of glucose adopted in the latter experiment was 2 % instead of 1 % whereby a greater rate of fermentation was secured (*vide* Exp. 3). This also exaggerates the difference between continuous and intermittent shaking. The results are shown graphically in Fig. 1 *c*.

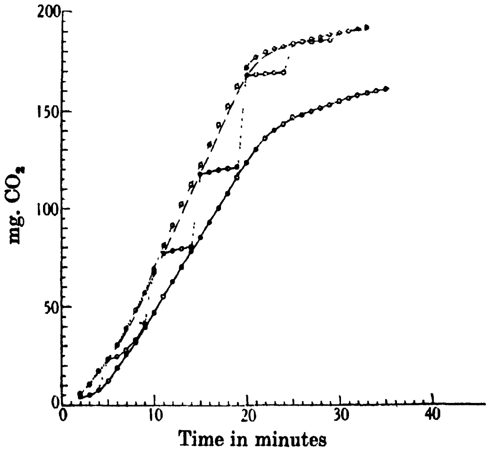

Fig. 1*b*.

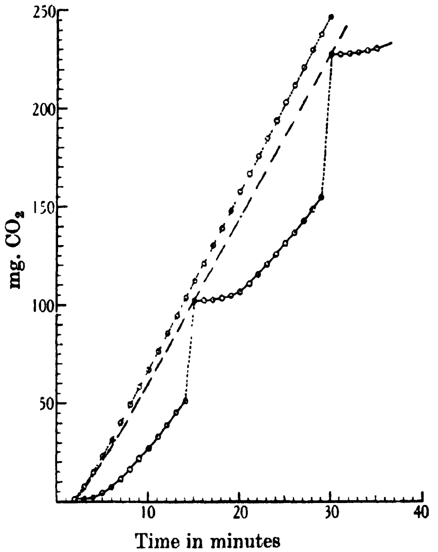

Fig. 1*c*.

Fig. 1*b*. -⊙—⊙- Periods without shaking. -⊙···⊙- Periods with shaking.
—•—•— Apparent curve for conditions (*b*).

Fig. 1*c*. -⊙—⊙- Periods without shaking. -⊙···⊙- Periods with shaking.
—•—•— Apparent curve for conditions (*b*).

It will be seen that intermittent shaking with only short time intervals, Exp. 1 *b*, the conditions adopted by Slator, gives results almost identical with those obtained by continuous shaking at 70 shakes per minute. The fermentation is proceeding at about the same rate in each case; a certain amount of supersaturation occurs between the shakings, but after a short agitation the readings approximate to those obtained with continuous shaking. Far more serious errors are involved if continuous shaking at the rate of, *e.g.*, 25 per minute, is adopted (Exp. 1*a*). Apparently this rate is inadequate to prevent a certain amount of supersaturation. If conditions are exaggerated as in Exp. 1*c* the results obtained after each agitation indicate a somewhat low rate of fermentation.

Exp. 2. Variation of yeast rate.

Glucose 0·5 g. (1 %). Yeast 2, 3, 4, 5 g. respectively.
Temp. 30°. Total volume 50 ml.

Table II. *Progressive rates of fermentation expressed as mg.* CO_2 *per minute.*

Time (mins.) ...	1	2	3	4	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	90	100
2 g. yeast	—	1·30	2·34	—	3·43	3·51	3·48	3·43	3·38	3·38	—	3·15	—	2·99	—	2·62	—	1·98	—	1·30	0·86	0·52
3 g. „	—	2·60	4·42	—	5·07	5·20	5·20	5·20	4·94	4·42	3·90	3·51	3·07	2·60	2·13	1·69	1·30	0·78	0·26	—	—	—
4 g. „	—	—	5·59	6·63	6·97	6·97	7·02	6·36	5·66	4·94	4·16	3·48	2·73	2·00	1·25	0·52	—	—	—	—	—	—
5 g. „	0·78	—	6·11	—	8·71	8·71	7·67	6·65	5·49	4·68	3·90	2·96	1·95	1·09	—	—	—	—	—	—	—	—

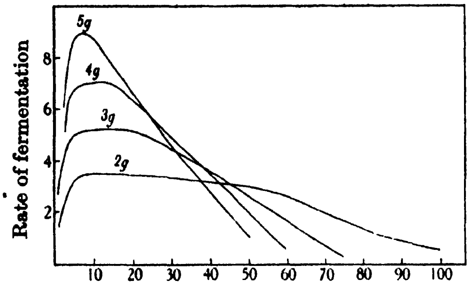

Fig. 2*a*.

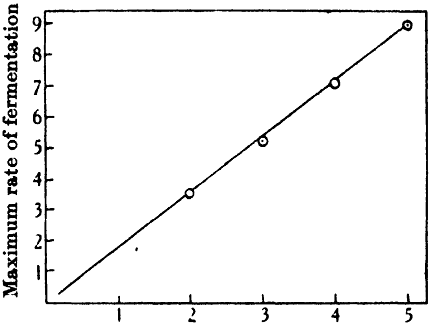

Fig. 2*b*.

The graph, Fig. 2*a*, shows the rate of fermentation plotted against time, and Fig. 2*b* the maximum rates plotted against yeast concentrations. It will be seen that the latter show exact proportionality.

Exp. 3a. Variation of initial glucose concentration.

Glucose 0·25–20 %. Yeast 4 g.
Temp. 30°. Total volume 40 ml.

Table III A. *Progressive rates of fermentation expressed as mg.* CO_2 *per minute.*

Time (mins.)...	1	2	3	4	5	8	10	15	20	25	30	35	40
0·25 %	2·29	2·80	—	—	3·57	3·31	2·80	1·43	0·25	—	—	—	—
0·50 „	2·55	3·31	—	—	4·13	—	3·97	3·16	2·14	1·17	—	—	—
1·0 „	3·31	4·08	4·84	5·10	5·25	—	5·20	4·74	4·28	—	—	—	—
2·0 „	—	4·84	5·35	—	5·81	—	5·86	5·81	5·61	5·25	5·10	—	—
5·0 „	—	—	—	—	6·02	—	6·37	6·37	6·37	6·24	6·12	5·99	5·86
10·0 „	—	—	—	—	6·37	—	6·84	7·04	7·14	7·14	7·14	7·14	7·19
20·0 „	—	—	—	—	5·10	—	5·56	5·61	5·66	5·71	5·71	5·76	5·86

In this experiment the yeast rate was the same as that employed by Nord and Weichherz. If the rates of fermentation are plotted against time it will be seen that with 0·25 % glucose the curve is typically compound exponential, but with increasing concentrations of glucose it flattens out and becomes quite flat with 5 % and 10 % glucose. With this yeast rate the highest maximum and constant rate was exhibited by the 10 % glucose solution and when at most only 0·6 % had been removed by fermentation.

Exp. 3 b.

Glucose 1·0–30 %. Yeast 2 g.
Temp. 30°. Total volume 40 ml.

Table III B. *Progressive rates of fermentation expressed as mg. CO_2 per minute.*

Time (mins.)...	5	10	15	20	25	30
1·0 %	3·16	3·21	3·21	3·16	2·96	2·80
2·0 „	3·21	3·62	3·67	3·52	3·47	3·31
5·0 „	3·42	3·82	3·95	4·05	4·05	4·05
10·0 „	4·18	4·31	4·31	4·31	4·33	4·33
15·0 „	3·31	3·87	3·87	3·87	3·90	3·90
20·0 „	2·55	3·06	3·26	3·31	3·31	3·36
30·0 „	1·02	1·33	1·53	1·58	1·63	1·66

Exp. 3 c.

Glucose 1·0–30 %. Yeast 2 g.
Temp. 30°. Total volume 80 ml.

Table III C. *Progressive rates of fermentation expressed as mg. CO_2 per minute.*

Time (mins.)...	5	10	15	20	25	30
1·0 %	3·01	3·56	3·61	3·61	3·50	3·28
2·0 „	3·50	4·10	4·10	4·10	4·05	3·72
5·0 „	3·78	4·54	4·82	4·82	4·82	4·82
10·0 „	4·38	4·93	4·93	4·93	4·93	4·98
15·0 „	4·24	4·44	4·44	4·44	4·49	4·49
20·0 „	2·74	3·12	3·12	3·12	3·18	3·18
30·0 „	1·37	1·53	1·64	1·70	1·70	1·75

The results of Exp. 3 *b* resemble those obtained in Exp. 3 *a*. The highest maximum and constant rate was apparently at a concentration of glucose approaching 10 %. In Exp. 3 *c* the yeast rate corresponded to that employed by Slator. At all concentrations of glucose from 1 % to 30 %, approximate constancy of rate of fermentation was observed over a period of time. By graphical procedure it can be seen that the concentration of glucose corresponding to maximum rate is about 6–8 %.

Exp. 3 *d.*

Glucose 0·1–15 %. Yeast 1 g.
Temp. 30°. Total volume 100 ml.

Table III D. *Constant rates of fermentation expressed as mg. CO_2 per minute.*

Initial concentration of glucose %	0·1	0·2	0·3	0·4	0·5	1·0	2·0	4·0	6·0	8·0	10·0	15·0
Rate of fermentation	0·71	1·10	1·31	1·44	1·55	1·77	1·95	2·10	2·17	2·12	2·05	1·85

(The progressive rates of fermentation in this experiment are stated in Part II, Exp. 2.)

With this low concentration of yeast the rate of fermentation became definitely constant for long periods of time. The maximum rate was exhibited by the solution of which the initial concentration of glucose was 6 %.

Exp. 3 e. Concentrations of glucose below 0·10 %.

For this experiment a different apparatus and procedure were required, as Slator's apparatus is not sufficiently sensitive. Indeed it was at the suggestion made privately by Dr Slator that this experiment was performed. The fermenting vessel and stirring arrangements employed were similar to those described by Nord and Weichherz [1929] and consisted of a 200 ml. flask with a stirrer passing through a mercury seal. The evolved CO_2 was swept out of the fermenting vessel by a current of CO_2-free air and passed through Pettenkofer tubes containing standard baryta. By the judicious use of two-way taps the CO_2 evolved was estimated at regular intervals by back-titration, the current of gas being diverted through a second tube while the first was being titrated and replaced. A control autofermentation was performed side by side with each experiment in an exactly similar apparatus, the fermenting vessel being immersed in the same thermostat, the appropriate deductions being made from each titration.

Table III E.

Glucose 0·04–0·08 %. Yeast 9 g.
Temp. 30°. Total volume 165 ml.
(Results in ml. 0·0944 *N* HCl.)

Initial conc. of glucose %	Time from addition of sugar mins.	Period of absorption of CO_2 mins.	Back-titration in fermentation tube ml.	Back-titration in autofermentation tube ml.	Difference ml.	Total fermentation of sugar ml.	Unimolecular velocity constant *k*
0·0848	10	10	28·9	33·1	4·2	4·2	0·0292
	20	10	25·5	38·4	12·9	19·1	0·0372
	30	10	31·8	36·3	4·5	21·6	0·0355
	40	10	35·9	37·35	1·45	23·05	0·0311
	50	10	37·65	38·75	1·1	24·15	0·0299
	60	10	37·85	38·35	0·5	24·65	0·0281
	70	10	38·4	38·7	0·3	24·95	0·0265
	80	10	38·8	39·1	0·3	25·25	0·0276
	100	20	38·8	39·0	0·2	25·45	—
0·0636	10	10	30·35	33·8	3·45	3·45	0·0288
	20	10	31·5	40·0	8·5	11·95	0·0323
	30	10	36·85	40·0	3·15	15·1	0·0291
	40	10	39·7	41·0	1·30	16·4	0·0253
	50	10	40·5	41·5	1·00	17·4	0·0239
	60	10	40·9	41·5	0·6	18·0	0·0227
	70	10	41·2	41·6	0·4	18·4	0·0219
	80	10	41·4	41·7	0·3	18·7	0·0217
	90	10	41·4	41·7	0·3	19·0	0·0238
0·0424	10	10	29·4	32·1	2·7	2·7	0·0414
	20	10	31·5	37·2	5·7	8·4	0·0372
	30	10	35·6	37·6	2·0	10·4	0·0327
	40	10	36·9	38·4	1·5	11·9	0·0354
	50	10	37·8	38·2	0·4	12·3	0·0332
	60	10	37·8	38·0	0·2	12·5	0·0311
	70	10	38·3	38·5	0·2	12·7	0·0337

Graphical inspection of the results given in Table III E indicated, as was anticipated, that the reactions were proceeding unimolecularly, provided that the induction period of about 7 minutes was neglected. To calculate the values of *k*, the constant, involved a little difficulty in deducting from the values of *t* the appropriate allowance for this induction period. This period could not be judged exactly enough from the graphs. The allowances made in the respective experiments, 7·3, 7·0 and 7·5 minutes, were judged by inspection. The reasonable constancy in the values of *k* justifies the allowances made and also the conclusion that at initial concentrations of glucose between 0·04 and 0·06 % if not also 0·08 %, the fermentation proceeds unimolecularly.

DISCUSSION.

The initial concentration of glucose corresponding to maximum constant rate of fermentation varies between 9 % or over in the case of the highest yeast rate (Exp. 3 *a*) and 6 % in the case of the lowest yeast rate (Exp. 3 *d*). With the low yeast rates, the rate of fermentation varies comparatively little between 2 % and 10 % glucose, whereas with the high yeast rates greater variations and more definite maxima are exhibited.

The results of Exp. 3 *d* lend themselves to mathematical treatment in two ways.

(1) *Theory of enzyme action of Michaelis and Menten* [*1913*].

Assuming a single enzyme action to limit the rate of fermentation within the yeast cell, application of the theory gives us the relationship

$$\frac{1}{v}=\frac{K_m}{V}\cdot\frac{1}{x}+\frac{1}{V},$$

where v = velocity of breakdown of enzyme-substrate compound, *i.e.* velocity of fermentation,

V = value of v when the enzyme is fully saturated with substrate and wholly combined with it,

K_m = dissociation constant of the enzyme-substrate compound (ES),

x = concentration of substrate.

In this equation V and K_m are constants. Hence if the fermentation conforms to the equation, the graph obtained by plotting $\frac{1}{v}$ against $\frac{1}{x}$ should be a straight line. In Table IV are tabulated values of x, v, $\frac{1}{x}$ and $\frac{1}{v}$ from the data in Table III D and interpolations from a graph drawn from them.

Table IV.

x = conc. %	v = rate	$1/x$	$1/v$
0·2	1·09	5·0	0·917
0·4	1·44	2·5	0·695
0·6	1·62	1·67	0·617
0·8	1·72	1·25	0·582
1·0	1·77	1·00	0·565
1·2	1·83	0·83	0·547
1·4	1·87	0·71	0·535
1·6	1·90	0·63	0·526
1·8	1·92	0·56	0·521
2·0	1·95	0·50	0·513
2·5	2·00	0·40	0·500
3·0	2·04	0·33	0·490
3·5	2·07	0·29	0·483
4·0	2·10	0·25	0·476
4·5	2·12	0·22	0·472
5·0	2·14	0·20	0·468
8·0	2·12	0·125	0·472
10·0	2·05	0·10	0·488

The results are plotted in Fig. 3 *a*. Between the limits 0·2 % and 5·0 % the graph is a straight line. From this it may be deduced that the Michaelis and

Menten theory holds for alcoholic fermentation of glucose within this range of conditions.

According to Haldane [1930] if an enzyme action follows the Michaelis and Menten equation three cases can arise.

(*a*) The substrate concentration remains so high until very near the end of the reaction that the enzyme is fully saturated.

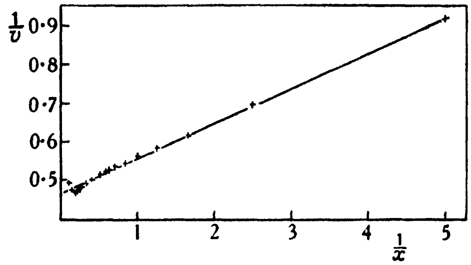

Fig. 3*a*.

Here, if the products of the reaction do not inhibit, y (the amount of substrate transformed in time t) $= Vt$. The curve for 2 g. yeast (Fig. 2 *a*) illustrates this case.

This is actually the condition under which Slator examined fermentation kinetics, and under which it is also easy to understand that the rate of fermentation is proportional to the concentration of yeast.

(*b*) The substrate concentration is so low that the amount of enzyme combined is proportional to the substrate concentration. Here

$$dy/dt=(a-y)\ V/K_m,$$

i.e.

$$t=K_m/V\,.\log_e\,(a/a-y)$$

$$(a=\text{initial substrate concentration}),$$

and the reaction is unimolecular. These are the conditions investigated in Exp. 3 *e*.

(*c*) The substrate concentration varies so that neither of these conditions is fulfilled.

Here

$$dy/dt=(a-y)\ V/(K_m+(a-y)),$$

therefore

$$\begin{aligned} Vt&=\int_0^y((a-y)+K_m)/(a-y)\,.dy\\ &=y+K_m\,.\log_e\,(a/(a-y))\\ &=y\left(1+\frac{K_m}{a}\right)+y^2\frac{K_m}{2a^2}+y^3\frac{K_m}{3a^3}+\dots\end{aligned}$$

If a is large compared with K_m the logarithmic term is at first negligible and the reaction proceeds uniformly, slowing down when y becomes appreciable. These are the conditions investigated by Nord and Weichherz. The curves for 5 and 4 g. yeast (Fig. 2 *a*) illustrate this case.

Hence, by application of the Michaelis and Menten theory, the results of Nord and Weichherz and those of Slator, apparently contradictory in part, are reconciled.

(2) *Mathematical treatment adopted by Slator.*

According to Slator the fermentation of sugars by yeast could be represented by the equation

$$\frac{d\,[CO_2]}{dt}=k\ [\text{conc. of sugar}]^n$$

(the yeast factor being kept constant),

or $$v = k\,[\text{sugar}]^n$$

i.e. $$\log v = \log k + n \log\,[\text{sugar}].$$

By plotting $\log v$ against log [sugar] we can, from the slope of the curve produced, derive the value of n. From the data of Exp. 3 *d* the values of $\log v$ and log [sugar] are tabulated in the following table (V) (first two columns) and plotted in Fig. 3*b*. From the slope of the curve are calculated the values of n tabulated in the fourth column, corresponding to concentrations in the third column, and plotted in Fig. 3 *c*.

Table V.

log (concentration) (log [sugar])	log (rate of fermentation) (log v)	Concentration %	n
-0·699	0·037	—	—
-0·523	0·117	0·30	0·381
-0·398	0·158	0·40	0·323
-0·301	0·187	0·50	0·265
0	0·248	1·0	0·167
0·301	0·290	2·0	0·125
0·602	0·322	4·0	0·054
0·778	0·328	6·0	-0·022
0·903	0·326	8·0	-0·071
1·0	0·312	10·0	-0·13
1·176	0·274	—	—

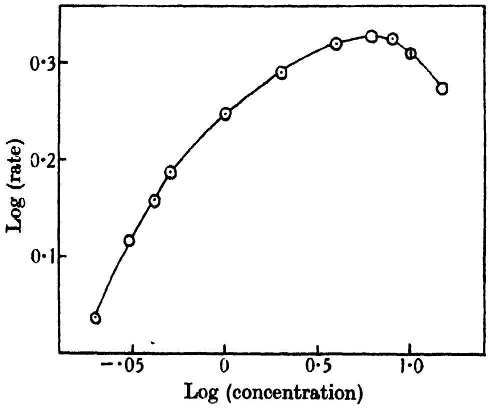

Fig. 3*b*.

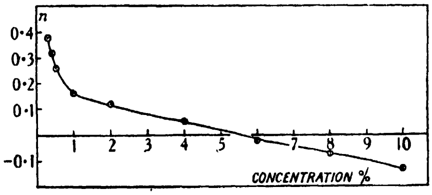

Fig. 3*c*.

It can be seen from Fig. 3 *c* that between the concentrations 1 % and 10 % of glucose the value of n approaches zero, *i.e.* the "approximate independence" of Slator holds good. At very low concentrations of sugar, however, the value of n approaches unity and the reaction becomes one of the first order with respect to sugar (*cf.* Exp. 3 *e*), which has been explained on the basis of the Michaelis and Menten theory.

SUMMARY.

1. It has been shown that if all the conditions which influence the rate of alcoholic fermentation of glucose by living yeast are carefully considered the kinetics are precisely similar to those of an ordinary enzyme reaction. The fermentation conforms to the theory of Michaelis and Menten and the mathematical deductions made from the theory apply under their special conditions.

Thus the apparently contradictory results of Slator and of Nord and Weichherz were obtained from experiments investigating respectively two special cases out of three possible cases under the theory. Relatively low concentration of yeast leads to saturation of the enzyme and constancy of reaction velocity over a wide range of substrate concentration (Slator). Relatively high concentration of yeast and sufficiently low concentration of sugar, 0·04–0·07 % glucose in the case investigated, yield a unimolecular reaction. Conditions intermediate between these yield a maximum rate of reaction followed sooner or later by a slowing down (Nord and Weichherz). In all cases an induction period precedes the attainment of the maximum rate.

2. The rate, if not also the nature, of the stirring or shaking is important, particularly when high yeast rates are employed. To attain uniform results shaking was preferred to stirring and was adopted whenever possible, from 45 to 70 shakes per minute being adequate. Slower shaking, *e.g.* 25 shakes per minute, gave a low rate of fermentation. On the other hand, intermittent vigorous shaking gave good results provided that the intervals between the shakes were not more than three or four minutes in duration.

3. In the case of glucose concentration as low as 1 % proportionality between yeast concentration and rate of fermentation was observed over a wide range.

REFERENCES.

Brown (1892). *J. Chem. Soc.* **61**, 369.
Haldane (1930). Enzymes. (Longmans, Green and Co., London.)
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Nord and Weichherz (1929). *Z. Elektrochem.* **35**, 612.
Slator (1906). *J. Chem. Soc.* **89**, 128.
—— (1908). *J. Chem. Soc.* **93**, 217.
—— and Sand (1910). *J. Chem. Soc.* **97**, 922.
Weichherz (1929). *Z. physikal. Chem.* A **145**, 330.
—— (1931). *Biochem. Z.* **238**, 325.

CXII. THE KINETICS OF ALCOHOLIC FERMENTATION OF SUGARS BY BREWER'S YEAST.

II. THE RELATIVE RATES OF FERMENTATION OF GLUCOSE AND FRUCTOSE.

By REGINALD HAYDN HOPKINS
AND RICHARD HENRY ROBERTS.

From the Department of Industrial Fermentation, University of Birmingham.

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The rate of fermentation of fructose by brewer's yeast was found by Slator [1908] to be the same as that of glucose over the range of conditions investigated. Hopkins [1931], however, observed that in low concentrations, less than 0·5 %, fructose was fermented appreciably more slowly than glucose at 30°. It was decided to extend the investigations on the effects of yeast rate and sugar concentration respectively (Exps. 2 and 3 reported in Part I of this series) to fructose, under conditions which permitted of comparison with glucose.

Experimental.

The apparatus and technique employed were exactly as described in Part I. Washed pressed yeast from the same source was used, and in Exp. 2 the glucose and fructose were fermented successively with yeast from the same sample.

Exp. 1. Variation of yeast rate.

Fructose 0·5 g. (1 %). Yeast 0·5–5·0 g.
Temp. 30°. Total volume 50 ml.

Table I. *Maximum rate of fermentation of fructose.*

Rates expressed as mg. CO_2 per minute.

Yeast rate (g.)	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0
Maximum rate of fermentation	0·68	1·53	2·05	2·78	3·48	4·06	4·63	5·07	5·49	5·80

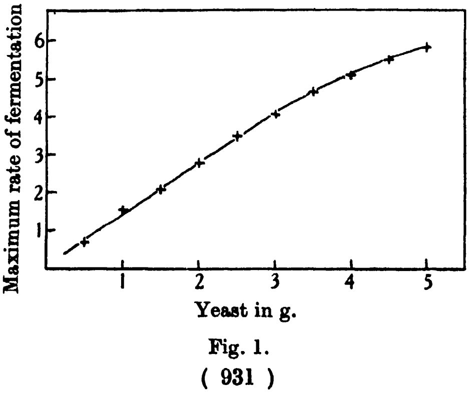

Fig. 1.

The graph, Fig. 1, shows proportionality between rate of fermentation and concentration of yeast only up to 3 g. in 50 ml., above which the fermentation rate is less than the proportional rate. This difference between the behaviour of fructose and that of glucose, which exhibited proportionality up to higher yeast rates, suggests that the yeast enzyme has a greater affinity for glucose than for fructose.

Exp. 2. Variation of initial concentration of glucose and fructose.

Glucose or fructose 0·1–15 %. Yeast 1 g.
Temp. 30°. Total volume 100 ml.

The rates of fermentation in solutions of various initial concentrations are set out in Table II. The results given correspond to certain arbitrary time stages and the constant rates calculated from the manometer readings over the period of approximate constancy are summarised in Table II *a*.

Table II. *Progressive rates of fermentation of glucose and fructose at varying initial concentrations.*

Rates expressed as mg. CO_2 per minute.

	Time (mins.)...	5	10	20	30	40	50
Glucose	0·1 %	—	0·74	0·62	0·59	—	—
	0·2 ,,	1·07	1·07	1·09	1·09	—	—
	0·3 ,,	1·13	1·22	1·33	1·36	—	—
	0·4 ,,	—	1·41	1·41	1·47	—	—
	0·5 ,,	—	1·44	1·55	1·58	—	—
	1·0 ,,	—	1·58	1·58	1·75	1·81	1·77
	2·0 ,,	—	1·41	1·58	1·93	1·98	1·98
	4·0 ,,	—	—	—	2·06	2·09	2·15
	6·0 ,,	—	—	1·81	2·04	2·15	2·17
	8·0 ,,	—	—	1·75	2·06	2·09	2·18
	10·0 ,,	—	—	1·92	2·04	2·04	2·05
	15·0 ,,	—	—	1·70	1·74	1·93	1·98
Fructose	0·1 ,,	—	0·48	0·45	0·42	—	—
	0·2 ,,	—	0·79	0·71	0·68	—	—
	0·3 ,,	—	0·85	0·91	0·91	—	—
	0·4 ,,	—	1·07	1·02	1·02	—	—
	0·5 ,,	—	1·19	1·24	1·16	—	—
	1·0 ,,	—	1·41	1·58	1·58	—	—
	2·0 ,,	1·58	1·64	1·78	1·78	—	—
	4·0 ,,	1·65	1·91	1·98	1·98	—	—
	6·0 ,,	1·60	1·77	1·87	2·01	1·98	—
	8·0 ,,	1·65	1·84	1·98	2·07	2·12	—
	10·0 ,,	1·56	1·56	1·77	1·77	—	—
	15·0 ,,	1·13	1·41	1·44	1·41	—	—

Table II*a*. *Constant rates of fermentation of glucose and fructose at varying initial concentrations.*

Rates expressed as mg. CO_2 per minute.

Concentration of sugar %	0·1	0·2	0·3	0·4	0·5	1·0	2·0	4·0	6·0	8·0	10·0	15·0
Rate, glucose	0·71	1·09	1·31	1·44	1·55	1·77	1·95	2·10	2·17	2·12	2·05	1·85
Rate, fructose	0·45	0·71	0·90	1·05	1·15	1·55	1·78	1·94	1·96	2·00	1·77	1·42

The results in Table II *a* are recorded graphically in Fig. 2 in which rates of fermentation are plotted as ordinates and concentrations as abscissae.

The results confirm those of Slator [1906] and Hopkins [1931]. Maximum rates of fermentation were obtained in solutions in the neighbourhood of 6 % initial concentration, and it is to be observed that whilst the maximum rates for the respective sugars differ but little, nevertheless the fructose curve lies below that of glucose throughout its length.

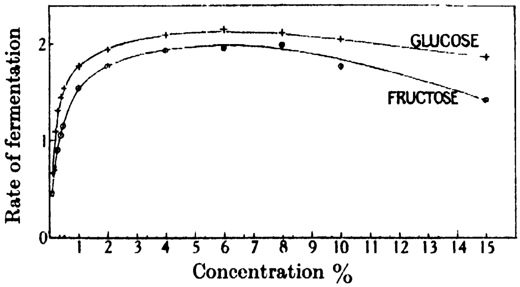

Fig. 2.

Discussion.

Application of the Michaelis and Menten [1913] equation,

$$\frac{1}{v}=\frac{K_m}{V}\cdot\frac{1}{x}+\frac{1}{V}$$

(see Part I), to the fructose results yielded the values recorded in Table III and plotted in Fig. 3 *a*.

Table III. *Fructose.*

x = conc. %	v = rate	$1/x$	$1/v$
0·2	0·71	5·00	1·408
0·4	1·06	2·50	0·943
0·6	1·27	1·67	0·787
0·8	1·43	1·25	0·699
1·0	1·54	1·00	0·649
1·2	1·60	0·83	0·625
1·4	1·66	0·71	0·602
1·6	1·70	0·63	0·588
1·8	1·74	0·56	0·575
2·0	1·78	0·50	0·562
2·2	1·80	0·45	0·556
2·4	1·82	0·42	0·550
2·6	1·85	0·38	0·541
2·8	1·87	0·36	0·535
3·0	1·89	0·33	0·529
3·5	1·92	0·29	0·521
4·0	1·95	0·25	0·513
6·0	2·00	0·17	0·500
10·0	1·84	0·10	0·544
15·0	1·42	0·07	0·704

As with glucose, the results of which were similarly dealt with in Part I (Table IV, Fig. 3 *a*), the graph is a straight line over a large part of its length. In the case of fructose this is true between the abscissa values corresponding to 0·4 % and 6 % concentration, indicating that the theory is valid over this range. In the case of glucose the corresponding limits were 0·2 % and 5·0 %.

In the cases of both glucose and fructose, it is possible to deduce the values of V, the maximum velocity of fermentation, and of K_m, the dissociation constant of the enzyme-substrate compound.

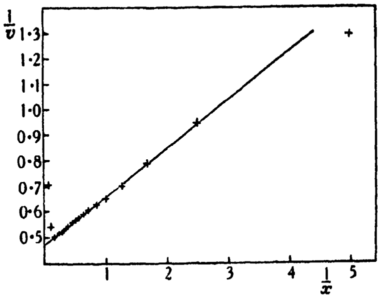

Fig. 3 *a*.

When x is large, *i.e.* when $\frac{1}{x}$ becomes zero, then

$$\frac{1}{v}=\frac{1}{V} \text{ and } v=V.$$

The value of $1/V$ can be read off from the graph where $1/x$ equals zero.

In the cases of both fructose and glucose the value of $1/V$ is the same and equals 0·47.

V is therefore 2·13.

Since K_m/V represents the slope of the curve, then

(*a*) Glucose

$$\text{Slope of line}=\frac{0\cdot92-0\cdot47}{5}=0\cdot090=\frac{K_m}{V}.$$

Hence $K_m=0\cdot090\times2\cdot13$

$=0\cdot192$, corresponding to $0\cdot01067M$.

(*b*) Fructose

$$\text{Slope of line}=\frac{1\cdot23-0\cdot47}{4}=0\cdot190\doteq\frac{K_m}{V}.$$

Hence $K_m=0\cdot190\times2\cdot13$

$=0\cdot405$, corresponding to $0\cdot0225M$.

These values of K_m, 0·192 and 0·405 for glucose and fructose respectively are in fairly close agreement with those deduced by Dawson [1932] from the results of Hopkins [1931], 0·2 and 0·4, assuming the applicability of the Michaelis and Menten theory to fermentation by living yeast.

The values of K_m and V have significance in that from them can be calculated the so-called "selectivity constant," *i.e.* that which is derived from the results of selective fermentations of mixtures of the two sugars, Hopkins [1931]. Thus it was shown by Michaelis and Menten [1913] that the relative rate of reaction of two substrates competing for the same enzyme is given by the equation

$$\frac{V_a}{V_b}\cdot\frac{K_a}{K_b}=K \text{ (selectivity constant)},$$

where K_a, K_b = the Michaelis constants of the two substrates, V_a, V_b = the theoretical maximum velocities with each substrate alone.

Substituting the values of V and K obtained above for glucose and fructose we get

$$K=\frac{2\cdot13}{2\cdot13}\cdot\frac{0\cdot405}{0\cdot192}=2\cdot10=K_{G/F},$$

which is again in close agreement with the value (2) calculated by Dawson from the experimental results of Hopkins.

The value of V, the maximum rate of fermentation deduced above (2·13, the number of mg. of CO_2 evolved per minute under the conditions of Exp. 2) is the same for both glucose and fructose. This means that for both sugars the theoretical maximum rate of fermentation is the same. In practice this is not attained since apparently hypertonic and other effects supervene.

Application of the equation, $\log v = \log k + n \log [\text{sugar}]$ as applied to fermentation of glucose (see Part I, Table V), gives the results in Table IV, columns 1 and 2, which are plotted in Fig. 3*b*. From the slope of the curve are calculated the values of n given in column 4, corresponding to the concentrations of fructose in column 3. These values are represented graphically in Fig. 3*c*. In both Figs. 3*b* and 3*c* the corresponding curves for glucose are reproduced for comparison (from Figs. 3*b* and 3*c* of Part I).

Table IV.

Log (concentration)	Log (rate of fermentation)	Concentration %	n
-0·699	-0·149	—	—
-0·523	-0·046	0·30	0·52
-0·398	0·021	0·40	0·49
-0·301	0·061	0·50	0·45
0	0·190	1·0	0·30
0·301	0·250	2·0	0·16
0·602	0·288	4·0	0·045
0·778	0·301	6·0	-0·035
0·903	0·292	8·0	-0·16
1·000	0·265	10·0	-0·30
1·176	0·152	—	—

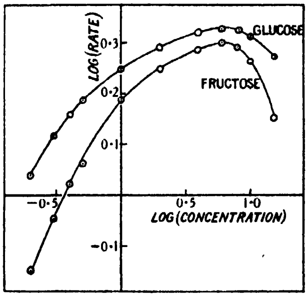

Fig. 3*b*.

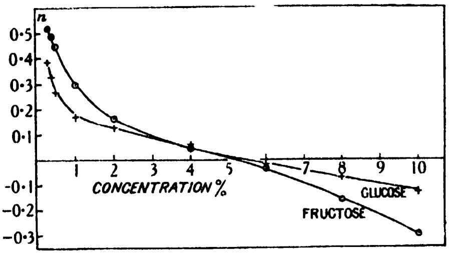

Fig. 3*c*.

Between concentrations of 2 and 8 % fructose the value of n approximates to zero, and the rate of fermentation as given by the equation $v = k [\text{fructose}]^n$ is approximately independent of the concentration of sugar. The corresponding limits for glucose concentration are 1 and 10 %.

Certain interesting differences between the behaviours of the two sugars are illustrated by these curves. The dependence of rate of fermentation on concentration of sugar is, at low values of the latter more marked in the case of fructose than of glucose. This is in agreement with the higher deduced value of K_m for fructose, for which sugar the yeast enzyme has less affinity than for glucose. This is confirmed by the results of the experiments on the effect of yeast rate. At high concentrations of sugar the rate of fermentation of fructose never quite equals that of glucose, since, apparently, hypertonic and/or other effects supervene. It is, however, evident that there exists a greater difference between the kinetics of fermentation of glucose and fructose than would be supposed from a superficial consideration of the results obtained by earlier workers.

SUMMARY.

1. Proportionality between the yeast rate and rate of fermentation of fructose exists up to a certain concentration of yeast, above which the fermentation rate is less than proportional. With glucose proportionality exists up to higher yeast rates.
2. Under selected identical conditions glucose is fermented at approximately the same rate between 1 and 10 %, and fructose between 2 and 8 % concentration, the rate for fructose being slightly less than that for glucose. Below these concentrations fructose is fermented much more slowly than glucose.
3. Under the conditions of experiment adopted, the theory of Michaelis and Menten is valid for the fermentation of glucose between 0·2 and 5 % and of fructose between 0·4 and 6 % concentration.
4. The values of the dissociation constants (K_m) of the compound of enzyme and substrate are 0·01067 and 0·0225 for glucose and fructose respectively, and the ratio of these gives the value for $K_{G/F} = 2{\cdot}10$ for the selectivity constant in agreement with the known value of this constant derived from experiments on selective fermentation of mixtures of the sugars by similar brewery yeasts.

REFERENCES.

Dawson (1932). *Biochem. J.* **26**, 531.
Hopkins (1931). *Biochem. J.* **25**, 1783.
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Slator (1906). *J. Chem. Soc.* **89**, 128.
—— (1908). *J. Chem. Soc.* **93**, 217.

CXIII. LACTOFLAVIN IN MICRO-ORGANISMS.

By LIONEL BRADLEY PETT[1].

From the Biochemical Institute, University of Stockholm, Sweden.

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In three years very rapid development has taken place in the study of the water-soluble, nitrogen-containing pigments, commonly called flavins or lyochromes, which occur in widely varying plant and animal tissues. In this time several members have been identified and their derivatives synthesised, especially by Karrer [1934, 1] and Kuhn [1934], lactoflavin has been suggested as identical with vitamin B_2 by György *et al.* [1934], and it has been shown in yeast to be bound to protein, forming an oxido-reduction enzyme, the "gelbes Ferment" of Warburg and Christian [1933]. As yet, however, almost nothing is known as to the method of production of lactoflavin in living material and the changes it undergoes. Although widely distributed in animals, it seems probable that flavin comes originally from plants. Lactoflavin has been found in yeast and in a few bacteria. The present work shows that yeast forms the flavin itself, that under various conditions it forms amounts of flavin different from normal, that some bacteria do not contain flavin, and that under various conditions blue-fluorescing substances may arise which are possibly related to flavin.

A review of early work on this subject by Wagner-Jauregg [1934] and preliminary reports by Pett [1935, 1, 2] should be consulted. The chemistry of lactoflavin may be briefly reviewed. Kuhn *et al.* [1933] obtained from milk a yellow-coloured, green-fluorescing substance which received the name lactoflavin, and around which the chief interest now centres. Lactoflavin has the formula $C_{17}H_{20}O_6N_4$. It easily accepts hydrogen thereby losing its colour and fluorescence. Oxygen from the air readily oxidises the reduced form. Under the influence of alkali and strong light it loses a carbohydrate group and the resulting yellow pigment, called lumilactoflavin, has the formula of 6:7:9-trimethyl*iso*alloxazine, which has been synthesised. The carbohydrate is probably in position 9. If lumilactoflavin is heated with alkali, urea is split off. A blue-fluorescing photo-derivative, 6:7-dimethylalloxazine, has been described by Karrer [1934, 2].

Flavin has been found in brewer's yeast in amounts varying between 18 and 30γ per g., and in slightly higher amounts in baker's yeast. In bacteria the amounts vary from the low value of 2γ per g. for *Thermobacterium helveticum* [Adler and Euler, 1934] and 10γ for *Bacterium pasteurianum* to the high values of 77–115γ for *Bact. delbrückii* and 91–136 for *Clostridium butyricum* [Warburg and Christian, 1933]. The present paper reports some further analyses for different organisms. Euler and Adler [1934, 1] showed that flavin in yeast is chiefly present in undialysable, or bound, form.

EXPERIMENTAL.

The yeast was grown in a medium containing per litre: 30 g. glucose, 5 g. $(NH_4)_2SO_4$, 3 g. Na_2HPO_4, H_2O, 3 g. KH_2PO_4, 2 g. NaCl, 0·1 g. $MgSO_4, 7H_2O$, and about 0·1 g. marmite; initial p_H about 6·5, final 4·5 to 5·0 unless controlled. To this control solution additions were made in the following amounts: urea,

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[1] Overseas Scholar (Canada) of the Royal Commission for the Exhibition of 1851.

10 g. per litre; $FeSO_4$, $FeCl_3$, 0·01 g. per litre; $CuSO_4,5H_2O$, 0·001 g. per litre; nucleic acid, 2 g. per litre; S, 3 g. per litre; Na_2S, 5 g. per litre. Cyanide and pyridine solutions, sterilised by filtration, were added after autoclaving the medium.

The yeast used for inoculation was a specially isolated strain of bottom yeast from a Stockholm brewery. Bacteriological technique was used throughout. An inoculum of 5 ml. per flask was used and contained about 1γ of flavin.

After maximum fermentation had been reached (about 5 days) the yeast was centrifuged and washed twice with distilled water. Part was then taken for moisture determinations and the remainder weighed in a small Erlenmeyer flask and treated with 6–10 ml. of 95 % ethyl alcohol. After some hours the mixture was heated at 70° for 2 minutes, under a condenser, and filtered. The yeast and filter were returned to the flask and re-extracted with a few ml. of 60 % alcohol. After 2–3 hours the mixture was again heated, filtered and re-extracted. If allowed to stand 12 hours or more the extract may be filtered without heating. Usually two treatments with 60 % alcohol were sufficient. The volume of the combined extracts was taken and a portion filtered through a hard paper. After one or more filtrations the solution was clear enough to be examined using a carbon arc lamp, the beams of which were concentrated by a lens. Comparison of the intensity of the green fluorescence is made with standard tubes of lactoflavin. Often a cloudiness prevents measurement, but is sometimes removed by shaking with light petroleum. This method is essentially that described by Euler and Adler [1934, 2].

More exact measurements, necessitated by the small amount of material available, were also made. The extracts were made alkaline, until equal to 0·5N NaOH, and exposed under a 500 watt lamp for 3 hours, using a special system of cooling and aeration. This solution was acidified to Congo red paper and extracted with chloroform. Several extractions remove the lumilactoflavin; the chloroform was then dried with sodium sulphate and filtered, and the fluorescence intensity was determined in a Zeiss "Stufenphotometer", previously calibrated by standard solutions.

All experiments were done two or more times. Results are expressed in γ of flavin per g. dry weight of substance. With any given solution the results usually agreed within ±3·0, but sometimes the error was about 6·0 %. Thus considerable differences are necessary to be significant.

RESULTS.

Flavin of yeast grown in different solutions.

The results shown in Table I indicate that the only method of influencing the amount of flavin was to affect the metabolism of the yeast, especially in relation to the physiological functioning of oxidation-reduction systems.

Table I. *The amounts of lactoflavin in yeast grown in different solutions.*

Flavin is expressed as γ per g. dry weight.

Control	32	*plus* Fe‴	32
Aerobic	17	*plus* Cu (p_H 4)	30
Anaerobic (vacuum) ...	40	(p_H 7)	33
plus malt (50 % of vol.) ...	20	*plus* urea, Cu	26
plus urea, Fe″	18	*plus* urea	28
plus Fe″ (p_H 4)	20	*plus* nucleic acid	29
(p_H 7)	24	*plus* sulphur	28
plus KCN (see Fig. 1) ...	65	*plus* sodium sulphide ...	32
plus cysteine	45	*plus* Cu, Fe″	40
plus active iron	19	Phosphate (see Fig. 3)	
plus pyridine (see Fig. 2) ...	50		

The results with cyanide, pyridine and phosphate are best seen from curves, such as those given in Figs. 1, 2 and 3.

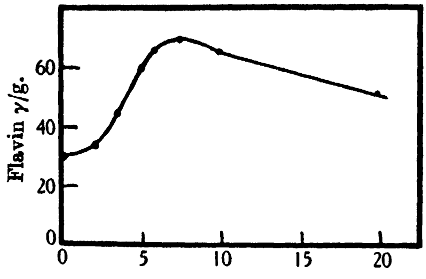

Fig. 1.

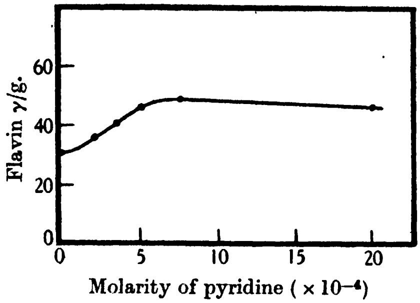

Fig. 2.

Fig. 1. The relation between the flavin content of yeast and the concentration of cyanide in which it was grown.

Fig. 2. The relation between the flavin content of yeast and the concentration of pyridine in which it was grown.

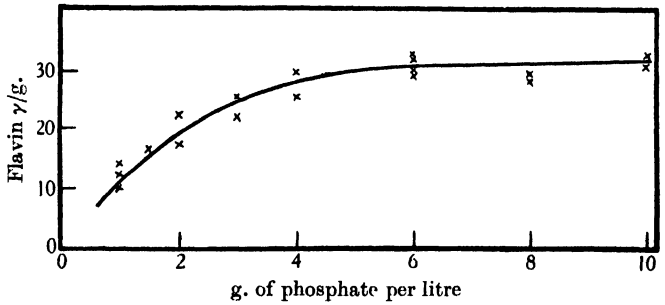

Fig. 3. The relation between the flavin content of yeast and the concentration of phosphate in which it was grown.

Discussion of flavin changes in yeast. Since only 2–3γ of flavin per flask were present at the start, and after some days' growth the yeast present contained 15γ or more, yeast must form flavin. The lower content of flavin resulting when yeast is grown with much malt extract suggests that favourable conditions for growth in the medium depress the development of flavin.

Under aerobic conditions, produced by a flow of air through the medium, the amount of flavin was lower than normal. Such conditions have long been known to depress fermentation and to promote reproduction and spore-formation. Since flavin was also depressed, some connection with fermentation is suggested.

Fig. 3 shows that a certain concentration of phosphate was necessary for normal flavin production. This seems clear in spite of some undetermined factor which caused considerable variation in the flavin contents of yeasts grown with little phosphate. This observation has great interest in view of the recent suggestion by Theorell [1934] that phosphate forms part of the active group of lactoflavin, when attached to protein to form the yellow enyzme. Since free phosphate was still present, the effect cannot be due to lack of phosphate. The explanation possibly lies in a lowering of the phosphatase activity of the yeast [Pett, 1934].

Of great interest is the increase of flavin in the presence of potassium cyanide, cysteine and pyridine (Table I, Figs. 1, 2). That flavin-enzyme has some kind of respiratory function was suggested as its properties became known in the original work of Warburg and Christian. It is, however, usually considered that iron-containing haemin compounds are chiefly concerned in cellular respiration, and the precise function of the yellow enzyme is still not clear, being possibly more related to fermentation. Nevertheless, when the normal, iron-containing oxidation mechanism is poisoned by the yeast being grown in cyanide, more flavin than normal is produced. Thus flavin can be considered an alternative system, developed to aid the cell's respiration, and it may be the only system left in cyanide-yeast.

Yeast grown in cyanide shows the characteristic spectrum bands of haemochromogen compounds, showing that these are present to some extent, even though not active. No evidence of utilisation of the cyanide by yeast was found: quantitative determinations at the beginning and end of several fermentations yielded about the same values.

Fermentation and respiration of cyanide-yeast.

For these experiments 5-litre flasks containing 4 litres of medium were used. Of the centrifuged yeast, washed free from KCN, part was used directly for flavin and moisture determinations, part was dried for dialysis in collodion, to determine the bound flavin, and part was suspended in water. This last portion was aerated according to the method of Ambrus *et al.* [1931], to remove easily oxidisable substances, and was then used for fermentation and respiration estimations and for Thunberg methylene blue reduction tests.

Fermentation was carried out, according to the well-known methods of Euler and Myrbäck, in small tubes using 0·5 ml. of 2 % KH_2PO_4 solution, 1 ml. of 10 % glucose and 0·5 ml. of yeast suspension. Results are expressed as ml. of gas evolved in one hour by 1·0 g. (dry) of yeast. The Thunberg test was performed with a final methylene blue concentration of $M/20{,}000$, and results are expressed in minutes required for decoloration, using fresh yeast equivalent to 0·01 g. The same amount of yeast was used for the Warburg respiration and results give ml. of oxygen per hour. Table II shows the results obtained with yeasts grown normally, with added iron and with added cyanide.

Table II. *Some properties of yeasts grown with added iron and cyanide.*

	Flavin					Thunberg MB reduction		
First experiment:	Total	Bound	Bound %	Fermentation	Warburg respiration	Without subst.	Glucose	Alcohol
Control	32	27	84	252	128	24	25	19
plus Fe	28	18	72	160	162	38	44	31
plus KCN	55	51	93	248	110	20	27	13
Second experiment:								
Control	30	25	81	170	190	—	—	—
plus Fe	26	21	81	118	246	—	—	—
plus KCN	46	44	95	178	140	—	—	—

The respiration of yeast grown in cyanide is somewhat less than normal but normal fermentation is maintained. The respiration was reduced from 140 only to 128 in the presence of $M/500$ KCN in the apparatus, indicating that the increased flavin content is chiefly responsible for the respiration observed, since under these conditions normal yeast respiration is inhibited 70 to 90 % [see Dixon and Elliott, 1929].

The alternative action between iron-compounds and flavin is supported by the observation of Malkov [1933] that phosphates inhibit respiration and stimulate fermentation by forming insoluble iron compounds. The work of Elvehjem [1931] had already shown that yeast grown with iron in the solution has an increased cytochrome content and respiration, the latter being clearly indicated also in Table II. Elvehjem also indicated that the longer yeast is grown the less its fermentation is inhibited by cyanide in the Warburg apparatus, and that the cyanide-stable system is concerned in glucose metabolism. Kuhn and Meyer [1929] have, on the contrary, suggested that this system is concerned in fat, not carbohydrate, metabolism. Zuckerkandl and Messiner-Klebermann [1932] have suggested that the cyanide-insensitive system concerned in fermentation also contains iron. From our work it might appear that this cyanide-stable part is flavin.

Effect of continuous culture of yeast in KCN.

When yeast was grown in a medium containing $M/1000$ KCN apparently normal gas production took place. Also, as shown above, the lactoflavin content was higher than normal. If yeast was taken from such a flask and added to another, also containing KCN, the same effect was observed. But when these transferences were continued a yeast was obtained, usually after the third to fifth culture, giving an extract with a blue fluorescence. In some cases all the fluorescence was blue, but more commonly some green (lactoflavin) fluorescence was also present. This blue persisted in subsequent transferences. It was not reducible by hydrosulphite. Inoculation into medium free from cyanide brought the green fluorescence back to normal, though sometimes some blue was present in the first transference.

It seems possible that during long-continued growth in cyanide yeast develops a special mechanism involving a blue-fluorescing substance which may or may not be related to lactoflavin.

Flavin contents of various organisms.

An examination of the quantity of lactoflavin found in various yeasts and bacteria, as shown in Table III, shows that flavin is not as generally distributed in bacteria as in higher organisms. All determinations were made as previously described.

Table III. *The amounts of flavin in various organisms.*

	γ per g.		γ per g.
Bottom yeast	20–25	*Aspergillus*	Nil (blue)
Top yeast:		*Penicillium*	Nil (blue)
Baker's (Stockholm) ...	25	*Proteus vulgaris*	Nil
Race "M"	33	*Staphylococcus albus* ...	Nil (blue)
Race XII	38	*Bacterium coli* A	Nil (blue)
Wild yeast:		*Bacterium coli* B ... ... (pathogenic)	Trace <1
Torula utilis	27		
Mycoderma cerevisiae ...	12	*Bacterium aerogenes* ...	9·8
Pichia membranifaciens ...	30	*Bacillus subtilis*	7·5

The idea that flavin is an alternative to actively aerobic, iron-containing systems and that it has a relation to fermentation finds some support in Table III. Thus a weakly fermenting, strongly aerobic yeast like *Mycoderma* has a very low content of flavin. *Pichia* and top yeasts are aerobic, but strongly fermenting, and have higher amounts. *Torula* is not so strongly fermenting, but has a fairly high flavin content, possibly because it is not strongly aerobic and is said to have incomplete haemin systems.

Moulds, like *Aspergillus* and *Penicillium*, of which the species were unfortunately not known, although containing green-fluorescing substances, showed only blue fluorescence after the treatment for the preparation of lumilactoflavin. They are, of course, strongly aerobic, but according to Frei *et al.* [1934] they contain neither cytochrome nor haematin compounds in general.

Flavin and bacteria.

No systematic study of flavin distribution in bacteria, such as that made for haemin compounds by Frei *et al.* [1934], has been made, but the results in Table III, together with previously mentioned determinations, give some idea of the situation. Undoubtedly also many older observations of colours in bacteria, especially of yellow colours, are significant in this connection. While some of these are undoubtedly carotenoids, the work of Ingraham and Baumann [1934] shows that this is true only in aerobes. Anaerobes, such as *B. butyricus*, do not contain carotenoids, but contain much lactoflavin.

That many bacteria do not contain flavin is also evident. While flavin is undoubtedly present in many kinds of tissue, it is thus not universal. Some bacteria contain other respiration pigments, like the pyocyanin of Wrede and Strack [1929], or the chloraphin of Kögl *et al.* [1932].

The possibility that some bacteria not only do not contain flavin but can actually transform it was indicated by Pett [1935, 1]. In that communication it was pointed out that an organism had been isolated from a lactoflavin solution which had become blue-fluorescing; and that this organism if placed in very small amount in a flavin solution caused the disappearance of the green fluorescence, and the development of a blue fluorescence. A few more facts can be presented. The use of fresh centrifuged, washed bacteria has been found more convenient. Dried bacteria in extremely minute quantity also effect the change described above. Their activity is about the same from p_H 6·5 to 8 but falls off in the more acid and alkaline ranges. The blue-fluorescent substance, either extracted from bacteria or developed as above, and then purified a little by extractions, fluoresces between 330 and 370$m\mu$; some preparations showed a faint absorption band about 264$m\mu$.

Of the organisms mentioned in Table III only *Bact. coli* A and *Bact. aerogenes* can act like the organism described by Pett [1935, 1], but *Bact. coli* acts much more slowly, and *Bact. aerogenes* produces a blue fluorescence reducible by hydrosulphite.

DISCUSSION.

This work contributes to two interesting points in the study of flavins: (1) the function of lactoflavin and (2) the importance of blue-fluorescing substances possibly related to lactoflavin.

The function of flavin in yeast might seem to be similar to that of the many other intermediate carriers for the oxidation-reduction activities, such as cytochrome or glutathione, assisted by the fact that it is autoxidisable. The increase of flavin in yeast grown in cyanide, shown in this paper, suggests that it is an alternative mechanism to iron-containing catalysts, but it is known that most of the normal respiration of yeast is not effected through flavin. The evidence advanced for a function in fermentation rather than respiration is not very strong for yeast but receives considerable support by analogy with bacteria.

In considering the function of lactoflavin in bacteria, as well as yeast, it must be remembered that the transference of oxygen is slower by way of flavin than by way of haemin compounds. Thus any cell containing haemin would not use flavin-enzyme for the same purpose. Using a lactic acid organism, which contained no haemochromogen, Warburg and Christian observed simultaneously the respiration and the amount of reduced flavin. They considered their results to show that all the respiration occurred by means of flavin. However, the spectrographic method of estimating the oxygen-transporting enzyme has been criticised as unreliable by Keilin [1933]. Adler and Euler [1934] showed an increase in respiration of bacteria in the presence of flavin, and especially if cozymase was also present.

In considering these results there is involved the old problem of what distinction must be made between fermentation and respiration. The respiration of lactic acid bacteria in the presence of fermentable substrate is quite different from the respiration of the bacterial cells themselves [Davis, 1933; Bertho and Glück, 1932]. Stimulation of the respiration of lactic acid bacteria by methylene blue is observed only if fermentation is taking place, and this may be true of flavin. An association with cozymase is of interest in this connection, though this appears to be related simply to the dehydrogenase system [Euler and Adler, 1934, 3]. The fact that the highest content of lactoflavin is found in anaerobic bacteria may be related to the strong fermenting ability of these bacteria rather than to their anaerobicity.

The present paper has shown that some organisms do not contain flavin, that other organisms contain it in only very small amounts, and that these results can be related to the fermentative power of the organisms. It has also been shown that if haemochromogen compounds are poisoned, the flavin system can develop and carry on suitable respiration. The function of flavin is, therefore, still obscure, except in the latter specific case.

Blue-fluorescing substances, possibly related to flavin, have been indicated from time to time by most of the workers in this field. These observations are complicated by the fact that many normal cell constituents, such as proteins or nucleotides, give a blue fluorescence in ultra-violet radiation, though not so many with a carbon arc lamp as used in this work. Karrer [1934, 1, 2] has described certain chemically prepared flavin derivatives with blue fluorescence. The chief example, lumichrome, loses its colour in the presence of hydrosulphite. Other apparent derivatives [see Euler and Adler, 1934, 4] are stable to hydrosulphite. Reduction by hydrosulphite is probably not a good criterion of lactoflavin but is of interest.

Pett [1935, 2] showed that a blue fluorescence appears during autolysis of yeast and during starvation of yeast. The present paper reports a blue fluorescence developed in yeast after continuous culture in cyanide, as well as from bacterial sources. The explanation of these observations cannot at present be given, but blue-fluorescing substances related to lactoflavin seem to be a promising field for future work.

SUMMARY.

Using a standard nutrient solution with the addition of various substances an attempt was made to vary the lactoflavin content of bottom yeast. The chief effect was observed by influencing the metabolism of the yeast, especially the functioning of redox systems. The following observations were made:

1. The presence of cyanide, cysteine or pyridine, which can poison the iron-containing catalysts, produced a flavin content much higher than normal.

2. Yeast grown in cyanide has a normal fermentation but a respiration below normal.

3. A certain amount of phosphate must be present in the medium for normal flavin development.

4. Under strongly aerobic conditions, which depress fermentation, the amount of flavin is also lowered. Some other evidence of a relation to fermentation is adduced.

5. Continued subculture of yeast in media containing cyanide resulted in the development of a blue-fluorescing substance, which partially or wholly replaces flavin.

6. The flavin contents of various organisms are reported.

The results are discussed in relation to flavin function and to blue-fluorescing substances.

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REFERENCES.

Adler and Euler (1934). *Z. physiol. Chem.* **225**, 41.
Ambrus, Banga and Szent-Gyorgyi (1931). *Biochem. Z.* **240**, 473.
Bertho and Glück (1932). *Liebig's Ann.* **494**, 159.
Davis (1933). *Biochem. Z.* **265**, 90.
Dixon and Elliott (1929). *Biochem. J.* **23**, 812.
Elvehjem (1931). *J. Biol. Chem.* **90**, 111.
Euler and Adler (1934, 1). *K. Sv. Vet. Ak. Arkiv Kemi, Mineral. Geol.* B **11**, No. 28.
—— —— (1934, 2). *Z. physiol. Chem.* **223**, 105.
—— —— (1934, 3). *Z. physiol. Chem.* **226**, 195.
—— —— (1934, 4). *Z. physiol. Chem.* **228**, 1.
Frei, Riedmüller and Almasy (1934). *Biochem. Z.* **274**, 253.
György, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 241.
Ingraham and Baumann (1934). *J. Bact.* **27**, 25.
Karrer (1934, 1). *Helv. Chim. Acta*, **17**, 735, 1518.
—— (1934, 2). *Helv. Chim. Acta*, **17**, 1010.
Keilin (1933). *Nature*, **132**, 783.
Kögl, Tönnis and Groenewegen (1932). *Liebig's Ann.* **497**, 265.
Kuhn (1934). *Ber. deutsch. chem. Ges.* **67**, 1936.
—— and Meyer (1929). *Z. physiol. Chem.* **185**, 193.
—— György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1577.
Malkov (1933). *Biochem. Z.* **262**, 185.
Pett (1934). Ph.D. Thesis, University of Toronto, Canada.
—— (1935, 1). *Nature*, **135**, 36.
—— (1935, 2). *K. Sv. Vet. Ak. Arkiv Kemi, Mineral. Geol.* B **11**, No. 53.
Theorell (1934). *Biochem. Z.* **275**, 36.
Wagner-Jauregg (1934). *Z. angew. Chem.* **47**, 318.
Warburg and Christian (1933). *Biochem. Z.* **266**, 377.
Wrede and Strack (1929). *Ber. deutsch. chem. Ges.* **62** B, 205.
Zuckerkandl and Messiner-Klebermann (1932). *Biochem. Z.* **261**, 55.

CXIV. THE HEMICELLULOSES.

I. ALCOHOLIC SODIUM HYDROXIDE AS A PRETREATMENT TO EXTRACTION.

By ARTHUR GEOFFREY NORMAN.

From the Biochemistry Section, Rothamsted Experimental Station.

(*Received March 1st, 1935.*)

INTRODUCTION.

THE hemicelluloses of plant materials form a group of polysaccharides, the investigation of which presents many difficulties. Neither their rôle nor their genesis is at present understood, and yet, in mature materials, they may amount to as much as 30 % of the whole, and they rank next to cellulose in quantitative importance. The name hemicellulose, due originally to Schultze [1892], is unfortunate, as it implies a close relationship to cellulose which, so far as most types of hemicellulose are concerned, does not appear to be justifiable. Hawley and Norman [1932] have discussed the occurrence and properties of hemicelluloses of plant materials and woods and differentiated them into two main groups differing in structure, distribution and function. For that type of polysaccharide intimately associated with the cellulose they have suggested the name "cellulosan". In most sources examined the cellulosan has proved to be a xylan, save in the Gymnosperms where a mannan also is found. The larger group of hemicelluloses is, however, not associated with the cellulose, but forms the incrusting material on the cell wall. Almost invariably has this type of polysaccharide been found to be a polyuronide. The two main groups of hemicelluloses, therefore, are the cellulosans and the polyuronides. In these papers it is proposed to deal more particularly with the incrusting polyuronides. Researches on the cellulosans will appear separately.

The isolation of hemicelluloses, and in particular those of the polyuronide group, in a pure condition is not easy. Extraction must be made with dilute alkali under conditions that result also in the solution of lignin and proteins, and the final preparation obtained after acidification and precipitation with alcohol or acetone is almost invariably contaminated with lignin, though this is rarely determined. The estimation of this group is still very unsatisfactory and inaccurate. Hydrolytic methods are of little use owing to the instability of uronic acids in hot mineral acids. Preece [1931, 2] produced a scheme for the evaluation of hemicelluloses in boxwood, depending on direct isolation and weighing, and Buston [1934; 1935] extended this to such materials as grasses and leaves in which the protein content is much higher. All the hemicellulose fractions obtained have to be corrected for the presence of protein.

To minimise the amount of lignin likely to pass into the preparation, Norris and Preece [1930], in isolating the polyuronides from wheat bran, carried out a pretreatment of the material with 50 % alcohol containing 1 % NaOH. Two extractions were given under a reflux for 2 hours each. This same pretreatment was also used and was then followed by further extraction with hot neutral 50 % alcohol in other preparations [Preece, 1930; 1931, 1, 2]. It was apparently assumed that because hemicelluloses are insoluble in cold 50 % alcohol under acid conditions, they will also be equally insoluble in hot 50 % alcohol under alkaline conditions. Later, in putting forward his quantitative method for boxwood, Preece [1931, 2] recommended one treatment for 1½ hours with boiling 50 % alcohol containing 1 % NaOH, followed by two extractions with neutral alcohol. Buston [1934], in preparing the polyuronides from cocksfoot grass, modified this treatment, giving instead a cold extraction overnight with the same concentration of alcoholic soda, and in his quantitative method he gave a similar pretreatment in the cold for 24 hours. Though the extraction with alcoholic soda was designed originally to remove lignin, it also reduces the amount of protein carried through into the subsequent alkaline extract and consequently into the hemicellulose preparation.

In some work on the delignification of straws and similar materials the effect of extraction with alcoholic soda of this and other concentrations was determined. It was found that while the removal of lignin was but partial, the material sustained heavy losses which could only be ascribed to the hemicelluloses. The results obtained caused the effect of alcoholic soda to be examined in more detail and show that its use in the heat is inadvisable prior either to preparation or estimation of hemicelluloses, and that even in the cold it is not without action on this group.

EXPERIMENTAL.

(a) *Extractions with NaOH in various alcohol concentrations.*

One gram samples of oat straw (60 mesh) were extracted in duplicate with various concentrations of alcoholic NaOH. After thorough washing with alcohol of the same concentration, containing a little acetic acid, then with 50 % alcohol to remove sodium acetate and finally with 95 % alcohol, the residue was determined. The two extractions were bulked and ground up, prior to duplicate lignin determinations, after hydrolysis with 5 % H_2SO_4 as described by Norman and Jenkins [1933]. The results are given in Table I, from which it is apparent that the loss in lignin forms only a small part of the total loss. Since a mature material was concerned, the three constituents, cellulose, hemicelluloses and lignin, account for about 90 % of the whole, and inasmuch as it is unlikely that true cellulose would be affected, the inference was that hemicellulose material, either polyuronide or cellulosan, or both, was extensively removed. As mentioned previously, no method for the estimation of the polyuronides is really satisfactory and accordingly no attempt at direct estimation was made, reliance being placed instead on the yield of furfuraldehyde as a measure of the hemicellulose present. The furfuraldehyde due to cellulosan may readily be determined and the difference between that and total furfuraldehyde represents that from the polyuronides.

Table I. *Extraction of oat straw (A) with alcoholic NaOH.*

Initial lignin content 11·39 %.
Lignin contents expressed on original material.

NaOH concentration %	Alcohol concentration %	Time in hours	Temperature	Residue %	Lignin content %	Delignification %
1	90	1	B.P.	56·9	4·61	59·6
1	90	¼	B.P.	62·9	4·74	58·4
1	90	2	60°	63·4	6·57	42·2
1	90	2	15°	70·9	8·07	29·2
0·2	90	1	B.P.	61·8	6·41	43·7
0·02	90	1	B.P.	75·8	9·84	13·6
1	60	1	B.P.	57·4	3·75	66·8
5	60	1	B.P.	57·2	3·92	65·3

A series of experiments was carried out on another sample of oat straw which proved to be rather less susceptible to alcoholic soda. Extractions were made with 100 ml. of a solution of 1 % NaOH in alcohol concentrations of 90 %, 70 %, 50 %, 30 % and with 1 % of NaOH in water. Each was carried out in duplicate and both hot and cold. The former were made at the boiling-point of the mixture for 1 hour and the latter at approximately 16° for 2 hours. The alcoholic soda extract in each case was acidified with H_2SO_4 and diluted so that the final volume was 600 ml. and the acid concentration 3 %. This was then hydrolysed by boiling for 2 hours, and the lignin, which remained insoluble, was filtered off, dried and weighed. The residue, after appropriate washing, was weighed, the duplicates bulked together, ground up finely and analysed. Cellulose determinations were made by the method of Norman and Jenkins [1933], furfuraldehyde determinations by the usual phloroglucide procedure and lignin determinations by treatment with 72 % H_2SO_4 for 2 hours and subsequent hydrolysis after dilution to 3 %. The results obtained are summarised in Table II, all the analyses being calculated on the original material.

Table II. *Extraction of oat straw (B) with 1 % NaOH in various alcohol concentrations.*

Results expressed as % of original material.

	Residue	Lignin in extract	Cellulose	Furfurald. from cellulose	Xylan in cellulose	Total furfurald.	Furfurald. from poly-uronides	Lignin (ash-free)	Delignification %
Original straw	—	—	49·6	8·41	13·0	16·69	8·3	14·47	—
(A) *Hot extraction:*									
1 % NaOH in alcohol									
90 %	80·45	1·99	46·8	7·96	12·4	15·59	7·6	11·33	22
70 %	68·88	7·40	45·4	8·01	12·5	15·64	7·6	6·04	58
50 %	59·99	8·57	45·2	8·17	12·7	13·07	4·9	4·99	65
30 %	52·94	7·70	44·2	7·65	11·9	8·91	1·3	4·74	67
Water	49·27	9·18	43·0	6·14	9·6	8·45	2·3	3·86	73
(B) *Cold extraction:*									
1 % NaOH in alcohol									
90 %	85·98	1·19	48·1	8·03	12·5	16·19	8·2	14·63	—
70 %	80·86	0·95	48·3	8·97	13·9	16·70	7·7	13·58	6
50 %	75·70	2·46	48·6	8·52	13·2	15·63	7·1	11·91	18
30 %	72·62	2·73	48·9	8·99	14·0	13·97	5·0	10·58	27
Water	72·51	3·15	46·5	6·91	10·7	13·36	6·5	10·73	26

Considering first the hot extractions, it is seen that there was an appreciable fall in the yield of furfuraldehyde from both polyuronides and cellulosan with even the 90 % alcoholic soda, and that with the lower concentrations of 50 % and below, the apparent loss of the former was very extensive. The 30 % solution appeared, rather surprisingly, to remove more of the polyuronides than an aqueous solution, though less of the xylan in the cellulose was extracted. The 50 % solution, as recommended by Preece [1931, 2] for the lowering of the lignin content, did in fact remove about 65 %, but this was accompanied by roughly 40 % of the polyuronides, if the furfuraldehyde yield may be taken as a direct measure, and 5 % of the xylan.

In the cold, the action of alcoholic soda was less drastic and in 90 % alcohol 1 % NaOH removed very little either of the polyuronides or the lignin. With lower concentrations there was some loss of polyuronides, and again it was noted that the 30 % alcoholic solution caused a greater loss of this constituent than did the aqueous solution. The figures for xylan in cellulose were unaccountably high in this group of extractions, and no apparent loss occurred save in aqueous solution. Delignification was in no case very effective and did not exceed 26 %. The recovery of lignin from the extract did not appear to be quite quantitative.

From Table II as a whole it is quite clear that any treatment which brings about a considerable lowering of the lignin content also extracts appreciable quantities of polyuronide material.

The preliminary experiments given in Table I indicated that a NaOH concentration of much less than 1 % would extract considerable amounts of material from straw, and accordingly the losses with 0·1 % NaOH in various alcoholic solutions were determined for oat straw B.

Table III. *Extraction of oat straw B with 0·1 % NaOH in various alcohol concentrations for 1 hour at boiling-point.*

Alcohol %	Residue %
90	80·4
70	77·9
50	76·6
30	72·8
—	77·3

The loss with 0·1 % NaOH in 90 % alcohol was found to be almost identical with that caused by 1 % NaOH, but at lower concentrations of alcohol the more dilute alkali did not prove to be such an efficient extracting agent. The curious difference between the effect of the 30 % alcoholic and the aqueous solutions was more marked in this series, the total loss being greater in the slightly alcoholic solution. Detailed analyses were carried out on the residues from these two extractions (Table IV) and the results showed that the extra loss in alcoholic solution was due to each of the three chief extractable groups, cellulosan, lignin and polyuronides. With hot 1 % NaOH as given in Table II only the polyuronides showed a greater loss in 30 % alcohol than in water.

Table IV. *Analyses of oat straw B extracted with 0·1 % NaOH in 30 % alcoholic and aqueous solutions for 1 hour at boiling-point.*

Results expressed as % of original material.

	Residue	Cellulose	Furfurald. from cellulose	Xylan in cellulose	Total furfurald.	Furfurald. from poly-uronides	Lignin (ash-free)
Original straw	—	49·6	8·41	13·0	16·69	8·3	14·47
0·1 % NaOH in 30 % alcohol	72·82	45·0	7·66	11·9	14·49	6·8	11·26
0·1 % NaOH aqueous	77·31	47·8	8·05	12·5	15·40	7·3	12·83

(*b*) *Extraction of various materials with 1 % NaOH in 50 % alcohol.*

Because of the apparent difference in susceptibility noted between the two samples of oat straw employed, a survey was made on a number of plant materials of widely different composition. Extractions were carried out with 1 % NaOH in 50 % alcohol for 1½ hours at the boiling-point, as recommended by Preece [1931, 2] for quantitative work, and also overnight in the cold with the same reagent, as used by Buston [1934], with the minor, and probably unimportant, difference that the time of standing was 16 hours instead of 24 hours. The materials were oak wood, jute fibre, old bean straw, mature hay, wheat plants just before harvest, green wheat plants six weeks before harvest (1·4 % N), mature vetches (2·53 % N) and young green vetches (4·88 % N), thus giving a range from highly lignified to young green materials.

Analyses were carried out on the extracted residues as described before. The results are given in Table V.

Table V. *Extraction of various materials with 1 % NaOH in 50 % alcohol; hot extractions 1½ hours at boiling-point, cold extractions 16 hours at room temperature.*

Results expressed as % of original material.

Material	Treatment	Residue	Cellulose	Furfurald. from cellulose	Xylan in cellulose	Total furfurald.	Furfurald. from poly-uronides	Lignin after hydrol.	Deligni-fication %
Oak	—	—	53·9	9·21	14·3	13·37	4·2	22·26	—
	Cold	88·4	51·4	8·47	13·1	12·83	4·4	19·81	11
	Hot	79·6	51·0	7·81	12·1	11·35	3·5	17·59	21
Jute	—	—	70·2	5·72	8·8	9·82	4·1	11·65	—
	Cold	89·2	68·3	5·05	7·8	8·80	3·8	8·58	26
	Hot	76·0	61·0	5·04	7·8	7·97	2·9	6·52	44
Bean straw	—	—	42·0	5·82	9·0	10·92	5·1	12·68	—
	Cold	85·1	44·5	5·77	9·0	11·49	5·7	11·70	8
	Hot	70·1	10·4	4·88	7·6	8·56	3·7	10·06	21
Wheat plants (mature)	—	—	46·7	8·31	12·9	13·27	5·0	13·79	—
	Cold	77·4	45·4	8·94	13·9	13·86	4·9	8·77	36
	Hot	66·1	41·8	7·97	12·3	12·92	4·9	3·67	73
Vetches (mature)	—	—	31·8	4·04	6·3	11·52	7·5	14·33	—
	Cold	68·9	35·3	4·10	6·4	8·74	4·6	11·03	23
	Hot	58·3	29·0	3·72	5·8	7·54	3·8	7·59	47
Hay (mature)	—	—	37·7	6·19	9·6	12·06	5·9	9·81	—
	Cold	67·6	35·9	5·29	8·2	10·81	5·5	9·64	2
	Hot	50·7	33·6	5·26	8·1	8·68	3·4	4·85	51
Wheat plants (green)	—	—	38·4	6·74	10·4	11·74	5·0	9·99	—
	Cold	57·6	38·3	7·47	11·6	11·78	4·3	6·23	38
	Hot	44·9	34·1	6·07	9·4	9·51	3·4	2·27	77
Vetches (young)	—	—	23·7	1·94	3·0	5·71	3·8	11·23	—
	Cold	58·3	27·9	2·49	3·9	5·01	2·5	7·98	29
	Hot	33·6	19·2	1·84	2·9	3·53	1·7	3·41	70

The mature materials suffered less total loss than the younger tissues, as might have been expected. In the case of the latter, much of the material removed was protein in nature. The cold treatment was not very effective in the removal of lignin, the percentage delignification only exceeding a third in one case. In some materials, though not in all, there was a distinct fall in the furfuraldehyde from polyuronides as a result of the extraction in the cold. The amount of hemicellulose removed, as judged by this figure, seemed to be larger in the case of the immature materials than in the mature; there was also a loss of xylan in some cases, though certain discrepancies occurred in these analyses. The figures obtained after the hot extraction, on the other hand, indicated in every sample an appreciable loss of polyuronide material and a fall also in the xylan associated with the cellulose. Delignification, of course, was more effective under these conditions, particularly in the less mature materials. However, in no experiment was it complete, and in oak and bean it was far from extensive.

There is no doubt that if the procedure of Preece [1931, 2] be followed, and a hot extraction with 1 % NaOH in 50 % alcohol be given, prior to the extraction of hemicelluloses, the values for that group will be low to an extent which varies with the nature of the material. It happened that Preece [1931, 2] worked with boxwood in which the hemicelluloses seemed rather more resistant to the action of alcoholic soda than those of some of the materials investigated in this work. While the treatment in the cold adopted by Buston [1934] has not such a drastic effect, it cannot be said to be without action on the polyuronides in all cases.

(c) *Extraction of isolated hemicellulose with alcoholic NaOH.*

Hemicelluloses once isolated are known to be rather more easily soluble than when *in situ* in the plant, and for example, go slowly into solution on boiling with water and readily in dilute alkali. Despite this change in properties, it was thought to be worth while to determine the solubility of a preparation in alcoholic NaOH. A crude sample of hemicellulose A was prepared from oat straw by the usual means but omitting the pretreatment with 50 % alcoholic soda. Without attempt at purification a cream-coloured product was obtained with a furfuraldehyde yield of 37·0 % and a lignin content (determined by boiling with 3 % acid for 4 hours and weighing the residue as lignin) of 14·7 %, both on an ash-free basis. Corrected for the presence of lignin, the furfuraldehyde yield represents 43·4 %. Small samples were treated with 1 % NaOH in 50 % and 90 % alcohol, both hot and cold, the hot extractions being continued for 1½ hours and the cold for 16 hours. The residues were filtered off, weighed and ashed, and the filtrates in the case of the hot extractions were taken for furfuraldehyde determinations. The results are given in Table VI.

Table VI. *Extraction of isolated hemicellulose with alcoholic NaOH.*

Results given on ash-free basis.

1 % NaOH in alcohol	Hemicellulose taken mg.	Residue mg.	% extracted	Furfurald. on extract mg.
90 % hot	300·3	258·3	14·0	Trace
	293·6	265·1	9·8	Trace
90 % cold	299·4	300·9	—	—*
	298·6	289·8	2·9	—*
50 % hot	295·4	211·2	28·5	10·9
	303·2	220·8	27·2	9·7
50 % cold	303·9	263·8	13·2	—*
	300·4	264·4	12·0	—*

Not determined.

The process of isolation did not produce any apparent increase in susceptibility to attack by alcoholic soda. While the 90 % solution in the cold removed little or nothing, some material went into solution in the heat. This was largely no doubt contaminating lignin since only a trace of furfuraldehyde was given by the extract. With the 50 % solution more than a quarter dissolved on boiling and about half this amount in the cold. The quantities available were too small to make possible any investigation of the nature of the material removed. If it be assumed that the hot extraction with 50 % alcoholic solution removed all the lignin from the hemicellulose, then the hemicellulosic fraction also removed had a furfuraldehyde yield of about 25 %, significantly lower than that of the parent product. This suggests the possibility of some partial fractionation or fission. These experiments are only of a preliminary nature, and this point will receive further attention.

DISCUSSION.

In his paper on the composition of boxwood, Preece [1931, 2] noted that the 50 % alcoholic soda treatments removed in addition to lignin some furfuraldehyde-yielding material which he described as a "lignosaccharide," though he showed that it resembled the hemicelluloses in properties and composition. The chief demonstrable difference appeared to be that the "lignosaccharide" fraction was rather more resistant to hydrolysis than the main "free hemicellulose" fraction, though not very much more so than a portion of the latter. The experiments described here may be taken to support the existence of "lignosaccharides" if by that term is implied only hemicellulosic material going into solution in alcoholic soda. No evidence has however been adduced for the view that "lignosaccharides" represent an entirely new group of plant polysaccharides, and nothing in this work could be held to support such an opinion. The term seems unfortunate and likely to confuse even further the nomenclature of hemicelluloses. There does not appear to be any clear-cut fraction removable by 1 % NaOH in 50 % alcohol; the amount of material removed may be increased by heating or decreased by raising the alcohol content, and there is no indication that a different type of product is obtained in such cases. The behaviour of the polyuronides with this reagent seems to be precisely the same as that of lignin or the cellulosans, part of both of which is removable by 50 % alcohol.

The data presented make it plain that the employment of a pretreatment by boiling with 1 % NaOH in 50 % alcohol prior to a hemicellulose preparation will result in a lowering of the yield by an amount which may reach 50 %. As a preliminary to quantitative work this procedure is clearly inadmissible. In the cold its effect is less drastic and on mature materials seems to be slight. However, the effectiveness of such a cold treatment as a delignifying process is so low that it is hardly worth while carrying out. On young green materials cold alcoholic soda seems to produce some lowering of polyuronide material, and if it is desirable to give such a treatment as a means of removing much of the protein, the magnitude of the hemicellulose losses should be determined for each individual material.

If in the preparation of polyuronides purity rather than yield is the main object, there may be some justification for a pretreatment with hot alcoholic soda as a delignifying agent, though it has not been unquestionably shown that the product is wholly unchanged by this treatment. The separation of lignin and hemicellulose is at present under investigation, and preliminary results suggest that it may be better to revise the present procedure and to obtain by alkaline extraction an impure hemicellulose product contaminated with lignin, and then subsequently to remove the lignin by other means.

SUMMARY.

1. As a pretreatment to the preparation or direct estimation of hemicelluloses, an extraction of the material with 1 % NaOH in 50 % alcohol is employed as a delignifying process. The effect of this and other concentrations of alcoholic soda has been studied in this connection.

2. In the heat, 1 % NaOH in alcohol concentrations up to 90 % extracts a considerable amount of polyuronide material, the amount increasing as the alcohol concentration is decreased. In the cold, the action is less drastic in 70 and 90 % alcohol, but quite appreciable in 50 % and lower.

3. The polyuronide hemicelluloses of different materials vary in the degree of susceptibility to alcoholic soda. Those of immature tissues seem to be rather more easily removed than those of older lignified materials.

4. No indication has been obtained that the material removed by alcoholic soda represents a special group of the polyuronides.

REFERENCES.

Buston (1934). *Biochem. J.* **28**, 1028.
—— (1935). *Biochem. J.* **29**, 196.
Hawley and Norman (1932). *Ind. Eng. Chem.* **24**, 1190.
Norman and Jenkins (1933). *Biochem. J.* **27**, 818.
—— —— (1934). *Biochem. J.* **28**, 2147.
Norris and Preece (1930). *Biochem. J.* **24**, 59.
Preece (1930). *Biochem. J.* **24**, 973.
—— (1931, 1). *J. Inst. Brew.* **37**, 409.
—— (1931, 2). *Biochem. J.* **25**, 1304.
Schultze (1892). *Z. physiol. Chem.* **16**, 387.

CXV. STUDIES ON THE ANAEROBIC DECOMPOSITION OF PLANT MATERIALS.

II. SOME FACTORS INFLUENCING THE ANAEROBIC DECOMPOSITION OF RICE STRAW (*ORYZA SATIVA*).

By CADAMBI NARASIMHA ACHARYA.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden.

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In a previous communication dealing with the anaerobic decomposition of rice straw [Acharya, 1935], it was noted that several factors such as temperature and reaction of medium greatly influenced the rate and course of the decomposition. An examination of some of these factors is reported in the present paper.

The experimental procedure and methods of analysis adopted were the same as used previously [1935], except for the changes and modifications noted in the experiments described below.

Exp. 1. Influence of temperature. To 20 g. portions of rice straw were added a mixture of 2 g. $KHCO_3$ and 2 g. NH_4HCO_3 in order to neutralise the acids formed, 0·2 g. K_2HPO_4, 0·05 g. $MgSO_4,7H_2O$, 10 ml. of a 2 % extract of a rice soil and water to make up 200 ml. The bottles were stoppered and paraffined and after evacuation were incubated at the desired temperatures. At weekly intervals the accumulated gaseous products were tapped off into a Hempel's burette, measured and samples taken for analysis. The liquid and solid residues in the bottles were analysed at the end of 6 months, according to the procedure already described. The data obtained, calculated per 100 g. straw taken, are given in Table I.

Table I. *Decomposition of rice straw at different temperatures.*

Per 100 g. straw ...	20°	25°	30°	35°	40°	45°
Total gas production (CO_2 not corrected for carbonates)	ml.	ml.	ml.	ml.	ml.	ml.
At the end of 1 month, CO_2	Nil	605	2705	3010	2810	1110
,, ,, 1 ,, CH_4	Nil	Nil	20	35	40	65
,, ,, 2 months, CO_2	1005	2050	3390	4125	3065	2090
,, ,, 2 ,, CH_4	225	305	410	495	320	185
,, ,, 3 ,, CO_2	2385	3770	6220	6980	4980	2985
,, ,, 3 ,, CH_4	705	1460	3950	4350	2095	640
,, ,, 6 ,, total CO_2	4940	6640	10290	10685	7180	3515
At the end of 6 months, net CO_2 (corrected for carbonates)	1262	4775	9830	9847	5530	1232
At the end of 6 months, CH_4	1205	4530	9585	9710	5310	1090
At the end of 6 months	g.	g.	g.	g.	g.	g.
Loss of dry matter less ash	17·3	23·1	35·2	35·8	24·1	15·4
Net CO_2 liberated corrected for carbonates	2·49	9·44	19·44	19·46	10·93	2·44
Net CH_4 liberated	0·86	3·24	6·87	6·96	3·81	0·78
Acetic acid present	8·57	4·43	1·07	1·92	3·86	5·42
Butyric acid present	1·88	0·83	0·23	0·48	0·82	1·02
Total weight of products	13·80	17·94	27·61	28·82	19·42	9·66

As the aim of the present investigation was to extend it to a study of anaerobic decomposition in the soil, the examination of the changes was confined to those taking place between 20° and 45°. The data show that the optimum amount of decomposition, as judged by loss of dry matter and total weight of products obtained, occurs between 30° and 35°. Outside this range there is an accumulation of acids and an increasing retardation in gas production. Hence in the experiments described below the temperature was maintained within this range. The figures given in Table I also show that though the amount of decomposition varies considerably at the different temperatures, the proportion of the net volume of CO_2 evolved, after correction for that evolved from carbonates, to that of methane produced is roughly unity.

Exp. 2. Influence of reaction of medium. Ammonium carbonate equivalent to 1 % N on the straw and K_2HPO_4, $MgSO_4,7H_2O$, soil extract and water in the quantities specified in the previous experiment were added to 20 g. portions of rice straw. The initial reaction of the medium was adjusted to the desired level by additions of H_2SO_4 or NaOH. All p_H determinations were made colorimetrically with the Hellige apparatus. Twice a week in the initial stages and at weekly intervals later, the accumulated gases were tapped off and 0·5 ml. of the reaction liquid was taken out for analysis, without the admission of air, by fitting a capillary of known volume into the exit tube, inverting the bottle and carefully unscrewing the pinchcock till the capillary was filled. The sample taken was diluted and titrated colorimetrically from a micro-burette to the desired p_H and the equivalent amount of acid or alkali was added to the contents of the bottle. On account of the buffering action of the organic acids formed, the method was found to give a reliable control of the p_H at the time of operation correct to ±0·1. Owing to the rapid formation of acids in the early stages, however, difficulty was experienced in maintaining the p_H at the desired figure over a number of days, though an effort was made to keep to the approximate level by frequent checks and additions of alkali when necessary. It has already been noted that the use of buffers was rendered impossible on account of the high concentration of acids formed.

The data obtained (Table II) show that the maximum loss of dry matter occurs at about p_H 8, at which level there is also the maximum evolution of methane and the minimum accumulation of acids. Above and below this p_H there is an increasing tendency for an accumulation of acids and a retardation of methane production, showing that the initial stage of acid formation is much more tolerant of changes in the reaction of medium than is the second stage of methane production. Very little methane production takes place below p_H 6 and above p_H 9; the rate is optimum at p_H 8 and falls rapidly on either side of this value. The accumulation of acids at p_H 9–10 suggests a method for the conversion of straw into acids, mainly acetic and butyric, by anaerobic digestion

Table II. *Decomposition of rice straw at different* p_H *values.*

g. per 100 g. of straw

p_H ...	5	6	7	8	9	10
At the end of 6 months:						
Loss of dry matter less ash	15·2	25·3	35·5	56·6	50·5	48·2
CO_2 evolved and in solution	1·61	8·27	13·40	22·87	14·12	12·92
CH_4 evolved and in solution	0·14	2·43	3·75	6·94	3·67	1·39
Acetic acid present	5·35	3·57	3·13	2·11	10·85	16·05
Butyric acid present	1·89	1·02	0·85	Nil	1·76	3·21
Total weight of products	8·99	15·29	21·13	31·92	30·40	33·57

at this range. For gas production a range of p_H 7·5 to 8 seems to be optimum. The concentration of CO_2 in solution is high at the higher p_H levels which may be due to the chemical action of alkali at high concentrations on rice straw.

Exp. 3. Influence of different proportions of water. According to the hypothesis [Symons and Buswell, 1933; Boruff and Buswell, 1934; Tarvin and Buswell, 1934] that anaerobic degradation is an oxidation-reduction reaction involving the addition of water and scission of the molecule into simpler compounds, a higher proportion of water would probably be necessary than if water played no part in the decomposition. Langwell and Lymn [1932] noted that a mobile liquid medium was necessary for successful thermophilic digestion of plant materials. A concentration of 8–10 % solids has been found optimum for the anaerobic digestion of sewage solids and garbage [Keefer and Kratz, 1934]. It was therefore thought interesting to examine the effect of adding different proportions of water on the rate of decomposition of rice straw.

The experimental details were the same as in Exps. 1 and 2, except that the ratio of straw to water was varied to give the following proportions: 1 : 4, 1 : 6, 1 : 8, 1 : 10, 1 : 15 and 1 : 20.

The results obtained (Table III) show that gas production is much impeded below a straw : water ratio of 1 : 8. The optimum concentration appears to be about 1 : 10, above which further additions of water are of no advantage. On the other hand, at a concentration of 1 : 20 there is actually a decrease in gas production. The optimum proportion of straw to water of 1 : 10 may be compared with the proportion of 1 : 4 or 5 generally found to be favourable for aerobic decomposition.

Table III. *Decomposition of rice straw with different proportions of water.*

Per 100 g. straw

	Straw : water ratio					
	1 : 4	1 : 6	1 : 8	1 : 10	1 : 15	1 : 20
Total evolution of gas (CO_2 not corrected for carbonates)	ml.	ml.	ml.	ml.	ml.	ml.
At the end of 1 month, CO_2	Nil	Nil	175	1080	1890	1585
,, ,, 1 ,, CH_4	Nil	Nil	35	980	1105	925
,, ,, 2 months, CO_2	545	1245	3625	5585	4865	3860
,, ,, 2 ,, CH_4	105	350	3900	5225	4670	3695
,, ,, 3 ,, CO_2	1830	3590	6095	7220	6930	5620
,, ,, 3 ,, CH_4	135	1520	5775	7030	6855	5335
,, ,, 6 ,, total CO_2	3865	8340	10525	11020	10985	10785
,, ,, 6 ,, net CO_2 (corrected for carbonates)	461	7266	10126	11020	10985	9833
At the end of 6 months, CH_4	325	6975	9945	11185	11210	10015
At the end of 6 months:	g.	g.	g.	g.	g.	g.
Loss in dry matter less ash	16·2	28·4	35·8	37·6	38·2	38·6
Net CO_2 (corrected for carbonates)	0·91	14·36	20·01	21·79	21·72	19·44
Net CH_4	0·24	5·00	7·13	8·02	8·04	7·18
Acetic acid present	7·56	2·29	1·07	Nil	Nil	2·06
Butyric acid present	2·30	0·86	Nil	Nil	Nil	0·72
Total weight of products	11·01	22·51	28·21	29·81	29·76	29·40

Exp. 4. Effect of inoculation with soil. The object of this experiment was to examine the differences if any between the rate and course of decomposition in presence of soil extract and in its absence. It is well known that natural products, *e.g.* straw, carry a microflora able to bring about their rapid decomposition. The experimental details were the same as before but no soil extract was added.

The data (Table IV) show that the rate and course of fermentation are the same both in presence and absence of soil inoculant, proving that the straw itself carries the micro-organisms necessary to bring about its decomposition.

Exp. 5. Effect of addition of phosphate. The present sample of straw contained 0·42 % P_2O_5 and the question as to whether addition of phosphate assisted anaerobic decomposition was examined by fermenting samples with and without the addition of phosphate (1 g. K_2HPO_4 per 100 g. straw).

The results (Table IV) show that the phosphate requirement for anaerobic digestion is quite small and is apparently satisfied by the phosphate present in the straw itself. Additions of phosphate, *e.g.* K_2HPO_4, do not exert any stimulating effect except by way of improving the reaction of the medium where the initial reaction is unfavourable for anaerobic digestion. Excess of phosphate at concentrations of 5 % and above on the straw taken was found greatly to impede the decomposition.

Table IV. *Exps. 4, 5, 6 and 7.*

Results in g. per 100 g. straw.

After 6 months:	Control	Exp. 4 Omission of soil inoculum	Exp. 5 Omission of phosphate	Exp. 6 Powdered straw	Exp. 7 Inoculation with vigorous culture
Loss of dry matter less ash	35·2	36·8	35·6	33·1	37·6
Net CO_2 (corrected for carbonates)	19·44	19·72	18·78	16·86	19·98
Net CH_4 evolved	6·87	7·10	6·52	5·82	7·32
Butyric acid present	0·23	Nil	0·86	0·98	Nil
Acetic acid present	1·07	0·83	1·82	3·22	0·42
Total weight of products	27·61	27·65	27·98	26·88	27·72
Preliminary lag period (days)	12	10	12	14	12

Exp. 6. Influence of powdering the straw (Table IV). This was tested by using 20 g. portions of straw ground so as to pass a 60-mesh sieve. The powdered sample in presence of excess of water formed a compact layer at the bottom, which did not ferment so readily as when chaffed straw was used. The total amount of decomposition is of the same order as with chaffed straw, but more acid is present. The amount of methane evolved is 8110 ml. as compared with 9585 ml. in the control.

Exp. 7. Effect of inoculation with an old culture (Table IV). A period of preliminary incubation or lag period is generally associated with anaerobic digestion and is attributed to the time taken for the development of the specific microflora necessary for effecting decomposition. The object of the present experiment was to see whether the lag period could be shortened by the addition to the medium of a small quantity of an inoculant from a vigorously growing culture. To 20 g. portions of rice straw, 5 ml. of the inoculant from an actively fermenting culture were added in place of the soil extract. The results show that the addition of such a small quantity of inoculant has no appreciable effect in shortening the period of incubation or hastening the rate of decomposition. It has been found that a fairly high proportion of seed is necessary to carry on successful digestion of fresh sewage solids [Whitehead and O'Shaughnessy, 1931].

Exp. 8. Use of different neutralising agents (Table V). Attention has already been drawn to the large amount of acids formed and the difficulty found in maintaining an alkaline reaction by the use of buffers. As the aim was to keep the reaction at about p_H 7·5 to 8, various weak neutralising agents were tried.

Table V. *Decomposition of rice straw in presence of different neutralising agents.*

g. per 100 g. straw after 6 months.

	Neutralising agent	Loss of dry matter less ash	Net CO_2	Net CH_4	Acetic acid	Butyric acid	Total weight of products
1.	$CaCO_3$, no N added	18·6	1·15	0·22	8·62	2·82	12·81
2.	Ammonium carbonate	35·2	19·44	6·87	1·07	0·23	27·61
3.	$NaNO_3$	31·8	16·01	4·19	3·64	Nil	23·84
4.	$NaHCO_3$, no N added	26·2	13·62	4·96	1·32	Nil	19·90
5.	$KHCO_3$, no N added	33·4	17·82	6·21	2·46	0·82	27·31
6.	NH_4HCO_3	34·6	18·62	6·46	2·26	0·84	28·18
7.	$CaCO_3 + NaHCO_3$, no N added	30·4	15·42	5·41	2·92	1·04	24·79
8.	$CaCO_3 + NH_4HCO_3$	31·8	16·26	5·61	3·40	1·12	26·39
9.	$NH_4HCO_3 + KHCO_3$	34·6	19·16	6·42	1·92	0·60	28·10
10.	$NH_4HCO_3 + NaHCO_3$	36·1	17·66	6·24	2·86	0·96	27·72

The figures show that $CaCO_3$ alone is not a satisfactory neutralising agent but it may give good results in combination with other reagents such as NH_4HCO_3, $KHCO_3$ or $NaHCO_3$. By itself $NaHCO_3$ is probably too alkaline (p_H 8·4–8·5) and impedes decomposition; it is not definitely known, however, whether the effect is due to the p_H of the system or to the high concentration of the salt. A mixture of NH_4HCO_3 and $NaHCO_3$ has given good results, as has also a mixture of NH_4HCO_3 and $KHCO_3$. The use of ammonium carbonate and sodium nitrate as neutralising agents has been examined in a previous communication [1935]. The degree of decomposition obtained by the use of $KHCO_3$, $NaHCO_3$ and $NaHCO_3 + CaCO_3$, in each case without the addition of nitrogen, further confirms the low nitrogen requirement for anaerobic digestion.

The neutralising agents described above are useful in that they can be added in advance along with the material in sufficient amount to neutralise the acids formed and so lead to successful decomposition without having recourse to frequent adjustments of the reaction of the medium. The acids formed may of course be neutralised from time to time as already described in Exp. 2. The degree of decomposition and the products formed are about the same in both cases.

Exp. 9. Use of different sources of nitrogen (Table VI). The parts played by ammonium carbonate and sodium nitrate in influencing anaerobic decomposition have previously been considered. The aim of the present experiment was to examine whether other nitrogenous compounds, easily reducible to ammonia under anaerobic conditions, could replace ammonium carbonate as neutralising agents. For this purpose, urea, calcium cyanamide, caseinogen, blood-albumin and egg-albumin were tried. 2 g. of each were added to 20 g. of straw, no other neutralising agent being added. The other experimental details were as before.

Table VI. *Decomposition of rice straw in presence of different forms of nitrogen.*

g. per 100 g. straw after 6 months.

	Ammonium carbonate	$NaNO_3$	Urea	Cyanamide	Egg-albumin	Blood-albumin	Caseinogen
Loss of dry matter less ash	35·2	31·8	32·8	29·6	23·8	22·4	18·2
Net CO_2 evolved	19·44	16·01	15·61	13·46	3·46	2·58	1·22
Net CH_4 evolved	6·87	4·19	5·32	4·52	1·12	0·84	0·38
Acetic acid present	1·07	3·64	3·12	4·24	7·29	7·52	4·72
Butyric acid present	0·23	Nil	0·96	1·12	6·60	5·66	8·18
Total weight of products	27·61	23·84	25·01	23·34	18·47	16·60	14·50

The figures show that urea and cyanamide are in a different category from the proteins and more closely resemble ammonium carbonate in their ability to neutralise the acids formed from straw.

An interesting point is the high proportion of butyric acid to acetic acid in cultures containing proteins. Possibly the butyric acid is derived from the anaerobic degradation of the added protein. The proportion of butyric acid to acetic acid is comparatively low where straw is digested by itself or in presence of ammonium carbonate, sodium nitrate, urea or cyanamide.

Expt. 10. Influence of the volume of the culture vessel (Table VII). The volume of the available free space above an anaerobic culture medium may sometimes markedly influence the rate of decomposition, *e.g.* a small vessel may produce high concentrations of CO_2 which may be directly toxic to the bacterial population [Kliewe and Kindhäuser, 1933] or may indirectly impede their activity by lowering the p_H. The use of culture vessels of different capacities may, in the present case, also throw some light on the mechanism of formation of methane.

In order to examine these points samples of 20 g. of rice straw were packed in bottles of different sizes as specified in Table VII and the decomposition was allowed to proceed for a period of 6 months.

In series I, one of the exit tubes of the bottle (300 ml. capacity) was connected to a receiver containing a known amount of sodium hydroxide to absorb the CO_2 formed as soon as liberated. The gas accumulating in the system was therefore almost wholly methane and was tapped off once every week. In series II, 400 ml. bottles were used and the gaseous products were removed weekly but the sodium hydroxide attachment was omitted and hence the CO_2 evolved was in contact with the culture medium for a week. In series III, with 1000 ml. bottles, the accumulated gases were removed monthly. 4-litre bottles were used in series IV and all the gases evolved were allowed to remain until the end. A mixture of 2 g. NH_4HCO_3 and 2 g. $KHCO_3$ per 20 g. straw, was added to all the bottles so as to neutralise the acids formed and maintain an alkaline reaction throughout the experiment.

Table VII. *Decomposition of rice straw in bottles of different sizes.*

g. per 100 g. straw after 6 months.

	Series I 300 ml. bottles with NaOH receiver	Series II 400 ml. bottles	Series III 1000 ml. bottles	Series IV 4000 ml. bottles
Loss in dry matter less ash	34·2	35·4	36·1	36·8
Net CO_2 evolved	18·94	19·70	20·40	21·52
Net CH_4 evolved	6·71	7·15	7·46	7·61
Acetic acid present	1·87	1·62	1·52	0·92
Butyric acid present	0·82	0·58	0·26	0·24
Total weight of products	28·34	29·05	29·64	30·29
	ml.	ml.	ml.	ml.
Volume of CO_2 evolved corrected for carbonates	9580	9965	10320	10725
Volume of CH_4 evolved	9365	10115	10410	10610

The results obtained show that the size of the interacting vessel has comparatively little influence on the course of the decomposition, provided the reaction of the medium is properly maintained. In all cases about 10,000 ml. of methane and an equal amount of CO_2 are evolved, confirming the observation made in a previous communication that a particular sample of straw produces an approximately constant quantity of methane. This is ascribed to the formation of a definite amount of acids from straw which are later decomposed into CO_2 and CH_4. It is worth pointing out that in all the series the proportion of CO_2 to CH_4 is roughly unity even though in some cases the CO_2 was removed as soon as it was liberated while in others it was allowed to be in contact with the medium for as long as 6 months. This lends additional support to the above hypothesis regarding the formation of methane and is opposed to the view that the methane is formed by reduction of carbon dioxide.

DISCUSSION.

An examination of the above results shows that the mechanism of anaerobic degradation of straw offers several points in marked contrast to aerobic fermentation. The former is much more restricted in its tolerance of changes in reaction than the latter. This may be explained as being due to the differences in the micro-flora in the two cases. The decomposition under aerobic conditions is brought about by a wide group of different species of microorganisms including fungi, bacteria and actinomycetes, which exert their optimum activity at different p_H levels. Under anaerobic conditions, however, the population is probably wholly bacterial and is possibly restricted to a few types operating within a narrow range.

Of the two stages in the decomposition of straw, *viz.* the first of acid formation and the second of gas production, it is noteworthy that the former is more tolerant to changes in reaction than the latter. The production of methane is active only in the range of p_H 7·5–8·5, whereas acid formation can go on between p_H 6 and 10. Anaerobic digestion at p_H 9–10 results in a rapid formation of organic acids which are stored up as salts and hence this might form a convenient method for the conversion of straw into acids, principally acetic. As the speed of acid formation is much more rapid than of gas production and since it is possible to calculate from the amount of acids formed the potential methane-producing capacity of straw, digestion at the above p_H range might also act indirectly as a method for the rapid determination of the methane-producing capacity of raw materials like straw.

Another interesting point of difference between aerobic and anaerobic decomposition of straw lies in the much larger amount of water required for successful decomposition in the latter case. Whereas a proportion of straw to water of 1 : 4 or 1 : 5 is sufficient for aerobic decomposition, over twice this amount is necessary for successful anaerobic decomposition. Part of the extra water required in the latter case is probably used up in the chemical reactions involving the degradation of straw material into acids and gases, but part of it may serve also to dilute the acids and other products of the fermentation which at high concentrations impede the progress of the decomposition.

The low nitrogen requirement for anaerobic digestion as compared with aerobic decomposition has been examined in detail in a previous communication [1935].

The presence of an active microflora on the straw itself and the absence of the need for extraneous inoculation, such as soil extract, confirms the observations of previous workers [Fowler and Joshie, 1920; Langwell and Lymn, 1932; Boruff and Buswell, 1929]. The absence of any response to the addition of a small amount of inoculum from a vigorously fermenting culture does not apparently support the hypothesis that the initial lag period is due to the need for the development of the specific microflora necessary to start the decomposition. Further work on this point is required before any conclusion can be drawn.

The low requirement for phosphate in anaerobic digestion could, as in the case of the low nitrogen requirement, be explained by assuming that a portion of the phosphorus in the straw dissolves and undergoes a continuous cycle of bacterial synthesis and disintegration sufficient to meet the needs of the bacterial population; thus the phosphorus would not be locked up in comparatively unavailable forms as it is under aerobic conditions.

SUMMARY.

1. For the anaerobic digestion of rice straw by mesophilic organisms, a temperature range of 30 to 35° is found to be optimum.

2. The p_H of the medium exerts a controlling influence on the course of the fermentation. The preliminary stage of acid formation is more tolerant to changes of reaction, but gas formation is greatly impeded outside the range of p_H 7·5–8.

3. The water requirements for anaerobic digestion are much higher than for aerobic decomposition. A proportion of straw to water of 1 : 10 is found optimum for the former.

4. The organisms required to decompose the straw are present on the straw itself and further inoculation is unnecessary and does not shorten the preliminary lag period.

5. The phosphorus requirement of anaerobic digestion is satisfied by the amount contained in the straw itself (0·42 %). Additions of phosphate have no appreciable stimulating effect. Excess of phosphate actually depresses the rate of decomposition.

6. Finely ground straw does not ferment more quickly than chaffed straw; the rate of gas production is in fact slower.

7. Of the different reagents tried for neutralising the acids formed during anaerobic digestion, NH_4HCO_3 and $KHCO_3$ are the best; they could be used singly or better still in admixture. It is possible also to control the reaction of the medium by additions of alkali or acid from time to time. Urea or cyanamide can replace ammonium carbonate but proteins cannot.

8. The amounts of CO_2 and CH_4 produced are approximately the same irrespective of the volume of free space above the culture medium, provided the accumulated gases are removed from time to time so as not to produce high concentrations of CO_2 or lower the p_H of the medium.

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REFERENCES.

Acharya (1935). *Biochem. J.* **29**, 528.
Boruff and Buswell (1929). *Ind. Eng. Chem.* **21**, 1181.
—— —— (1934). *J. Amer. Chem. Soc.* **56**, 886.
Fowler and Joshie (1920). *J. Indian Inst. Sci.* **3**, 39.
Keefer and Kratz (1934). *Sewage Works J.* **6**, 14, 240.
Kliewe and Kindhäuser (1933). *Arch. Hyg.* **110**, 211.
Langwell and Lymn (1932). *J. Soc. Chem. Ind.* **51**, 988.
Symons and Buswell (1933). *J. Amer. Chem. Soc.* **55**, 2028.
Tarvin and Buswell (1934). *J. Amer. Chem. Soc.* **56**, 1751.
Whitehead and O'Shaughnessy (1931). *Proc. Inst. Civil Eng.* **233**, 38.

CXVI. THE HYDROLYSIS OF HEXOSEDIPHOSPHORIC ESTER BY BONE PHOSPHATASE.

II. (*a*) THE PARTICIPATION OF PHOSPHOHEXOKINASE; (*b*) THE ISOLATION OF PURE FRUCTOSE-I-PHOSPHATE[1].

BY BÉLA TANKÓ AND ROBERT ROBISON.

From the Biochemical Department, The Lister Institute, London.

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THE study of the hydrolysis of phosphoric esters by bone phosphatase may serve two purposes, to gain knowledge of the chemistry of the esters and to throw light on specific properties of the enzyme. The present investigations were planned with both these objects in view.

When hexosediphosphoric ester (fructose-1:6-diphosphate) is hydrolysed by acids, the two phosphate groups are split off at very unequal rates. The velocity constants for the reactions in *N* HCl at 100° have been determined as 52×10^{-3} for the group in position 1 and $4{\cdot}2 \times 10^{-3}$ for that in position 6 [Macleod and Robison, 1933], the latter value giving the velocity after the first group has been removed. The marked difference in behaviour of the two groups towards acids enabled Neuberg [1918], by partial hydrolysis of the diphosphoric ester, to obtain a fructosemonophosphate in which the phosphate group is now known to occupy position 6.

The relative rates of hydrolysis of phosphoric esters by bone phosphatase may differ widely from those of their hydrolysis by acids. By the action of this enzyme on hexosediphosphoric ester Macleod and Robison [1933] obtained the second fructosemonophosphate containing the phosphate group in position 1. They did not succeed in freeing this compound entirely from the Neuberg ester. Its isolation in pure condition is described in the present paper.

A point of much interest had emerged from earlier experiments [Martland and Robison, 1929], namely, that in the hydrolysis of fructosediphosphate by preparations of bone phosphatase, some of the sugar undergoes intramolecular change, producing a mixture of aldoses and ketoses; similar changes were also observed in the hydrolysis of glucosemonophosphate [Robison and King, 1931].

The more detailed investigations of Macleod and Robison [1933] appeared to confirm these findings. The proportion of aldose formed during hydrolysis of hexosediphosphate varied with the phosphatase preparation employed and with the experimental conditions; but no explanation of this variation was, at the time, forthcoming. By stopping the hydrolysis when 50 % of the phosphate had been set free they obtained, in addition to unchanged diphosphate and free sugar, a mixture of glucose-6-phosphate (possibly containing mannose-6-phosphate), fructose-6-phosphate, and a new ester characterised by its strong laevorotatory power and its very rapid hydrolysis in *N* HCl at 100°. From the hydrolysis curve of the mixed monophosphates it was calculated that the above esters were present in the approximate proportions 3 : 2 : 5. After removal of the aldosephosphates by oxidation with bromine and separation of the sparingly soluble barium phosphohexonates, the residual mixture contained 75 % of the new ester and 25 % of fructose-6-phosphate. Further purification was not attained at that time, but the properties of the new ester were calculated from those of the mixture and provided strong evidence that its constitution was that of fructose-1-phosphate. The suggestion was then made that the two phosphate groups of fructose-1:6-diphosphate are removed at equal rates by bone phosphatase but that, during removal of the group from position 1, intramolecular change occurs, resulting in the formation of a mixture of fructose-6-phosphate, glucose-6-phosphate and mannose-6-phosphate. These three esters would together constitute 50 % of the monophosphate fraction, the remaining 50 % being fructose-1-phosphate which could not undergo such intramolecular change.

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[1] A brief report of these results was communicated to the Hungarian Physiological Society in May 1934 and an abstract was published in *Ber. ges. Physiol.* (1934), 81, 563.

The main objects of the work now to be described were (*a*) the discovery of factors controlling the conversion of ketose into aldose derivatives, (*b*) the isolation of fructose-1-phosphate in pure condition and the further study of its properties.

EXPERIMENTAL.

The fructosediphosphate used in these experiments was prepared by allowing a mixture of 50 g. dried yeast, 30 g. fructose and 100 ml. H_2O to ferment at 25° with gradual addition of a phosphate-sugar solution ($0{\cdot}5\ M$ $Na_2HPO_4 + M$ fructose); the hexosephosphates were separated by the method previously described [Robison and Morgan, 1930]. The fructosediphosphate obtained from five fermentations was purified by repeated precipitation of the acid barium salt from its aqueous solution by alcohol [Macfarlane and Robison, 1935] until a small portion, after conversion into the neutral barium salt, gave satisfactory figures on analysis (Table III). The phosphatase was prepared by the method of Martland and Robison [1929].

The methods of procedure for the hydrolysis and for the separation and examination of the products were similar to those employed in the later experiments of Macleod and Robison [1933] with slight modifications. All solutions were prepared with water which had been freed from dissolved air by boiling. The hydrolysis was carried out at 28° in presence of chloroform and was stopped by addition of H_2SO_4 to lower the p_H to 5; organic and inorganic phosphates were precipitated with basic lead acetate[1]. The solution, after removal of lead as $PbSO_4$, was examined for the free sugars. The basic lead precipitate was decomposed with sulphuric acid and converted into barium salts, which were separated in the usual way.

It was first sought to discover whether the relative rates at which the two phosphate groups are set free or the extent to which the conversion of fructose into aldose occurs during hydrolysis could be influenced by such factors as differences in the proportion of phosphatase, the p_H of the solution, or the concentration of magnesium.

Controls. As controls for these experiments a series of 8 solutions was prepared containing sodium phosphate and fructose but no phosphoric ester; phosphatase was added to 4 of these. Two of each group were adjusted to p_H 7·0 and two to 8·4, while $MgSO_4$ was added to one of each pair. A similar series was also prepared containing glucose in place of fructose. The proportions of the various constituents were equivalent to those in Exp. 1. The solutions were kept at 28° during 10 days, estimations of total and inorganic phosphate and reducing power (Hagedorn and Jensen (H.J.) and iodimetric) being carried out at intervals. No evidence was obtained of esterification of phosphate or of significant change in the amount or character of the sugar present in any of the solutions. Two of each series (p_H 8·4, without $MgSO_4$, with and without phosphatase) were finally evaporated to dryness and the residues were extracted with boiling 85 % alcohol; over 95 % of the sugar originally present was recovered in each case. The sugars recovered from each of the two fructose solutions had $[\alpha]_{5461} - 103°$, calculated on the H.J. values, and I values of 4 %, calculated as glucose; those recovered from the glucose solutions had $[\alpha]_{5461} + 60°$ and I values of 98 %. Fructose and glucose have $[\alpha]_{5461} - 110·8°$ and $+62°$ respectively. It was thus evident that very little, if any, conversion of free sugar occurs under these experimental conditions.

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[1] The very alkaline solution "25 % $Pb_3O(C_2H_3O_2)_4$" (B.D.H.) was not used, since, as previously noted, it causes changes in the dissolved sugars.

Exp. 1. Rapid hydrolysis. A solution (140 ml.) of sodium fructosediphosphate, prepared from the acid barium salt, was divided into 4 equal portions, each containing 200 mg. P; two of these were brought to p_H 7·0 and two to p_H 8·6; magnesium sulphate was added to two of the solutions, one at each p_H, to give a final concentration of 0·005 *M*. 5 ml. of a phosphatase suspension containing 200 mg. of the dry preparation A_1 were added to each of the four solutions; the volume was made up to 40 ml., and the p_H was adjusted to 7·0 and 8·4 respectively; at intervals, portions were removed for analysis. Hydrolysis proceeded more rapidly than had been anticipated and had reached approximately 90 % in the solutions at p_H 8·4 when tested after 24 hours. In the two solutions at p_H 7·0 hydrolysis was stopped after 51 and 68 hours respectively, when slightly more than 50 % of the phosphate had been set free. The H.J. reducing power increased in all the solutions proportionately to the amount of hydrolysis. The initial iodimetric reducing power, equivalent to about 75 mg. aldose and mainly attributable to the phosphatase, was not significantly changed in any of the solutions. Conversion of ketose into aldose derivatives could not, therefore, have occurred to an appreciable extent during the hydrolysis. Analyses of the products are given in Table I; none showed more than a small iodimetric reducing power and it was concluded that the free sugars consisted almost wholly of fructose and that the monophosphate fractions contained over 90 % of the two fructose esters in approximately equal proportions. The differences found between the products of hydrolysis at p_H 7·0 and p_H 8·4 with and without added magnesium salt were not very marked or consistent.

Exp. 2. Slow hydrolysis. A further hydrolysis was carried out at p_H 8·4, using only 20 mg. phosphatase per 200 mg. organic P, *i.e.* one-tenth of the amount of phosphatase employed in the previous experiment. Hydrolysis reached 50 % in 19 days and was then stopped; the monophosphate fraction gave analytical values corresponding closely with those calculated for a mixture of fructose-6-phosphate and fructose-1-phosphate in equal proportions. The amount of aldosephosphate did not exceed 6 % and was probably less[1].

While the results of these experiments justified the conclusion that the two phosphate groups of fructosediphosphate are removed by bone phosphatase at nearly equal rates, they showed that the conversion of fructose into aldose, previously observed, is not a necessary consequence of this hydrolysis.

It seemed possible that an explanation of these varying results might lie in the difference in purity of the phosphatase used. Preparations obtained by addition of alcohol and ether to aqueous extracts of bone had been employed for most of the earlier hydrolyses; but the phosphatase A_1 used in the above experiments had been purified further by re-extracting the first precipitate with 30 % aqueous alcohol and again precipitating the phosphatase with alcohol and ether[1]. This process of purification might have brought about the destruction or removal of some agent present in the cruder phosphatase and responsible for the change in the molecule of fructose.

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[1] Fructosephosphates have a small iodimetric reducing power.

Further evidence on this point was obtained by hydrolysing fructosediphosphate with other preparations of purified bone phosphatase (Exps. 3 and 4) and with crude bone extract (Exp. 5). These hydrolyses were carried out at 28° and p_H 7·6–7·8, without addition of magnesium salt. Analyses of the products and descriptions of the phosphatase preparations are given in Table I.

Table I. *Products obtained from partial hydrolysis of fructosediphosphate by bone phosphatase.*

	Phosphatase			Conditions of hydrolysis				Sugars	Monophosphoric esters (barium salts)					
										Reducing power as glucose				
Exp.	Prep.	Amt.	Ester mg. P	p_H	$MgSO_4$ *M*	Duration days	Hydrolysis %	$[\alpha]_{5461}$ calc. on H.J. (°)	T.P. %	H.J. %	I %	Aldosephosphate %	Fructose Selivanoff %	$[\alpha]_{5461}$ (°)
1*a*	A_1	200 mg.	200	7·0	—	2·8	55	− 108	7·8	29	3·5	8	25	− 17·2
1*b*	A_1	200 ,,	200	7·0	0·005	2·1	52	− 105	7·8	30	3·5	8	25	− 16·3
1*c*	A_1	200 ,,	200	8·4	—	1	87	− 97	7·7	30	4·0	9	23	− 15·1
1*d*	A_1	200 ,,	200	8·4	0·005	1	92	− 99	7·6	30	4·5	10	22	− 14·4
2	A_1	20 ,,	200	8·4	—	19	50	− 97	7·8	30	2·9	6	23	− 17·5
3	B_1	45 ,,	155	7·7	—	4	50	− 103	7·8	28	3·0	6	26	− 18·7
4	C_2	45 ,,	155	7·7	—	4	50	− 103	7·8	28	3·2	7	25	− 18·1
5	D	12 ml.	—	7·7	—	2	50	− 53	7·8	29	14·4	32	19	− 13·3
6	D	200 ,,	3921	7·8	—	3	47	− 61	7·8	28	13·0	29	19	− 13·4
7	E	200 ,,	4500	7·6	—	7	48	− 55	7·8	29	14·7	32	18	− 12·9

Fructose has $[\alpha]_{5461}$ − 110·8°; $C_6H_{11}O_5PO_4Ba$ requires P 7·85 %.

Phosphatase preparations:

A, B, C, precipitates by addition of alcohol and ether to filtered aqueous extracts of rabbit bones.

A_1, purified by extraction with 30 % aqueous alcohol and reprecipitation with alcohol and ether in presence of potassium acetate.

B_1, purified by extraction with 50 % aqueous alcohol and reprecipitation with alcohol and ether.

C_2, purified twice by extraction with water and reprecipitation with alcohol in presence of sodium sulphate.

D, E, crude aqueous extracts of rabbit bones.

The monophosphate fraction produced by crude extract had an I value of 14·4 %, equivalent to 31 % of aldosemonophosphate; the free sugar appeared to contain about the same proportion of aldose[2]. The monophosphates obtained with purified phosphatase had I values of only 3 % and probably contained less than 6 % of aldosephosphate, while the proportion of aldose in the free sugars was equally low. Clearly, therefore, the crude bone extract contained an active agent which was not present in the purified phosphatase.

The phosphohexokinase of bone and marrow.

Presence of the active agent in bone marrow. In preparing the phosphatase most, but not all, of the marrow was removed before the bones were extracted with water. Tests were made with two extracts, one prepared from the heads of long bones without removing the marrow, the other from specially cleaned fragments of hard bone from the shafts. When allowed to act on hexosediphosphate both extracts produced monophosphate mixtures containing about 30 % of aldo-esters. Experiments were next carried out with (*a*) an aqueous extract of marrow and (*b*) the precipitate obtained from such an extract by addition of

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[1] A difficulty experienced in obtaining flocculation of the material at this stage was overcome by addition of potassium acetate.

[2] Calculated from the specific rotation, assuming fructose and glucose to be the principal components. The I values of these sugar fractions were vitiated by the presence of I-reducing substances derived from the crude enzyme preparation.

Table II. *Interconversion of aldo- and keto-hexosephosphates by preparations of bone phosphatase and marrow.*

			Phosphatase*		Marrow†									
Exp.	Substrate	Aldo. %	Prep.	mg./mg. P	Prep.	ml./mg. P	t°	p_H	Time hrs.	Hydr. %	Aldo. %	Time hrs.	Hydr. %	Aldo. %
10	Fructose-1:6-diphosphate	4	C	0·4	—	—	28	7·5	17	32	18	48	81	29
		4	C	0·4	*a*	0·01	28	7·4	17	32	24	48	70	36
	"	4	C	0·4	*a*	0·04	28	7·2	17	32	25	48	67	36
	"	4	C_2	0·9	—	—	28	7·5	17	27	4	48	64	6
	"	4	C_2	0·9	*a*	0·01	28	7·3	17	29	20	48	57	33
	"	4	C_2	0·9	*a*	0·04	28	7·2	17	30	24	48	59	35
11	Fructose-1:6-diphosphate	4	C	0·2	—	—	23	8·4	—	—	—	40	71	29
		4	C_2	0·4	—	—	23	8·4	—	—	—	40	63	5
						mg./mg. P								
	"	4	C_2	0·4	*b*	0·3	23	8·4	—	—	—	40	65	30
	"	4	C C_2	0·02 0·4	—	—	23	8·4	—	—	—	40	74	21
	Fructose-6-phosphate	5	C_2	1	*b*	1	23	8·4	—	—	—	40	98	57
	"	5	—	—	*b*	1	23	8·4	—	—	—	40	9	55
12	Fructose-6-phosphate	5	—	—	*b*	1	23	7·7	17	2	56	40	5	56
	Fructose + inorganic phosphate	< 1	—	—	*b*	1	23	7·7	17	—	< 1	40	—	< 1
13	Fructose-6-phosphate‡	5	—	—	*b*	3	23	6·4	4	< 1	61	20	2	62
	"	5	—	—	*b*	3	23	7·7	4	1	62	20	4	63
	"	5	—	—	*b*	9	23	7·7	4	2	62	20	7	62
	Fructose-1-phosphate	2	—	—	*b*	2	23	7·7	4	1	2	20	5	2
	"	2	C_2	6	*b*	2	23	7·7	4	82	2	20	100	2
	Glucose-6-phosphate	92	—	—	*b*	3	23	7·7	4	1	63	20	4	63

* Phosphatase preparations. C, precipitate obtained by addition of alcohol and ether to aqueous extract of rabbit bones. C_2, C twice purified by extraction with water and reprecipitation with alcohol in presence of sodium sulphate.

† Marrow preparations. *a*, aqueous extract of bone marrow; marrow from the long bones of young rabbits was shaken with 3 times its weight of water, left 3 hours at 0° and centrifuged; 0·04 ml. ≡ 10 mg. fresh marrow. *b*, precipitate obtained by addition of alcohol and ether to unfiltered aqueous extract of bone marrow; 1 mg. was obtained from 11·6 mg. fresh marrow.

The amounts of these preparations used are given as mg. or ml. per mg. P of the substrate.

The figures for aldose and aldo-derivatives are based on the I values (less the amount due to the enzyme preparations) and are given as percentages of the total calculated hexose (not the H.J. value) in the substrate. The fructose esters reduce I to a small extent and are, therefore, shown as apparently containing 2–5 % aldosephosphate; fructose (Kerfoot) gives an I value of about 0·8 %. The specimen of glucose-6-phosphate used contained a small proportion of other esters, chiefly mannosephosphate.

The concentrations of the substrates varied between 0·015 *M* and 0·04 *M* in the different experiments.

‡ In Exp. 13, tests on fructose-6-phosphate + marrow were also done after 40 minutes and gave nearly the same percentages of aldo-derivatives as were found after 4 hours.

alcohol and ether. The results are shown in Table II. It was found (Exps. 10, 11) that either of the marrow preparations, when added to the purified phosphatase C_2 restored the power to produce aldo-compounds from fructosediphosphate. Addition of the marrow extract to the cruder phosphatase C led to a slight increase in the percentage of aldo-compounds formed on hydrolysis of the diphosphate; but larger quantities of marrow extract, added to either crude or purified phosphatase, did not raise the proportion of aldo-compounds beyond certain limits, namely, about 25 % of the total hexose when hydrolysis had reached 32 % and about 36 % when 60–70 % of the phosphate had been set free. Other experiments, not quoted in Table II, showed that the marrow preparation, like the crude phosphatase, lost its power to effect this change when left for some hours in aqueous alcohol. Exp. 11 also showed that the addition to phosphatase C_2 of 1/20 its weight of phosphatase C was sufficient to increase the proportion of aldo-compounds from 5 to 21 %, while 10 times the quantity of C alone produced 29 %. It was concluded that the same agent was present in both marrow and bone extracts but the results did not warrant the assumption that its presence in the latter was due solely to the marrow left in the bone.

In order to learn more about the properties of this agent its action on other phosphoric esters and on fructose + inorganic phosphate was investigated. By the use of the marrow preparation *b*, whose phosphatase activity was very low, and the highly purified phosphatase C_2, the action of the two enzymes could be readily distinguished.

Fructose-6-phosphate was converted into aldo-esters to the extent of 55–63 % by marrow preparations alone at p_H 8·4, 7·7 and 6·4, very little hydrolysis taking place (Exps. 11, 12, 13). The amount of conversion was, in some cases, maximum within 40 minutes. When both phosphatase and marrow were allowed to act on this ester, 98 % hydrolysis occurred in 40 hours, but the percentage of aldose was the same as that produced by marrow alone.

Fructose-1-phosphate, on the other hand, did not yield any aldo-compounds with marrow alone (5 % hydrolysis) or with marrow + phosphatase (100 % hydrolysis), nor did the marrow preparation produce any aldose from a mixture of fructose + inorganic phosphate in periods up to 40 hours. The action of marrow on glucose-6-phosphate caused the aldose content to fall to 63 %, the same proportion as that produced from fructose-6-phosphate under similar conditions.

It should be stated that the amounts of aldosephosphates present in the equilibrium mixtures may be somewhat higher than the maximum values recorded, for two reasons; (*a*) owing to the relatively large I-reducing power of the enzyme preparations the total reduction exceeded in some cases the limit recommended for this method [Macleod and Robison, 1929]; (*b*) the aldo-esters probably included some mannose-6-phosphate which gives an abnormally low I value.

The results of these preliminary experiments suggest that the active agent present in bone and marrow is a phosphohexokinase having the power to convert the hexose-6-phosphates into an equilibrium mixture of aldo- and keto-esters. It has no action on fructose-1-phosphate and, most probably, none on intact fructose-1:6-diphosphate. When the diphosphate is hydrolysed by bone phosphatase, fructose-1-phosphate and fructose-6-phosphate are formed in approximately equal proportions, so that if the kinase is also present the percentage of aldo-compounds in the products may rise to about half that given by fructose-6-phosphate under optimum conditions. The actual figure will depend on the relative activities of the phosphatase and kinase, that is, on how much of the fructose-6-phosphate is hydrolysed before conversion. The kinase does not effect the conversion of free sugar.

This phosphohexokinase is probably identical with the enzyme discovered by Lohmann [1933] in extracts of muscle, kidney, liver, brain and dried yeast. Lohmann showed that these extracts converted the Neuberg ester into a true equilibrium mixture which resembled, in its rates of hydrolysis by HCl and in its aldose content, the Embden and Robison esters of muscle and yeast fermentations. The conversion was extremely rapid; at 20° equilibrium was reached in a few seconds with dialysed muscle extract and in about 5 or 10 minutes with the other tissue extracts. In our own experiments only a few tests were made after short periods, but these showed that fructose-6-phosphate was converted into the equilibrium mixture in about 40 minutes at 23° and p_H 7·7 by 3 mg. of marrow *b* ($\equiv$ 35 mg. fresh marrow) per mg. ester P. The proportion of animal tissue extract used by Lohmann corresponded with about 600 mg. fresh tissue per mg. ester P. It would seem, therefore, that the activity of the marrow is at least as great as that of brain, kidney and liver tissues.

Lohmann showed that a similar equilibrium was also reached in a few seconds when muscle extract was allowed to act on the aldosephosphate mixture resulting from partial hydrolysis of the Embden ester by acids. The same result was obtained in Exp. 13 of the present series, starting with glucose-6-phosphate. Other experiments [Patwardhan and Robison, 1935] have shown that mannose-6-phosphate behaves in the same way when acted on by yeast extract. There can therefore be little doubt that each of these hexose-6-phosphates is converted by phosphohexokinase into an equilibrium mixture in which all three esters are present. Each has been isolated from the hexosemonophosphate of fermentation which, however, contains other esters whose precise nature is still unknown. It would be interesting to discover whether the kinase has any effect on these also or plays any part in their formation. It has been suggested [Robison and Rosenheim, 1934] that the calcifying mechanism of bone may consist of a complex enzyme system analogous to those in yeast and muscle. The presence of phosphohexokinase in association with the phosphatase of hypertrophic cartilage and osteoid tissue may have some significance in this connection and its location in the bone is therefore being further investigated.

The isolation of fructose-1-phosphate.

The high laevorotation of the mixed hexosemonophosphates obtained with purified phosphatase suggested that these would provide the most suitable material for the preparation of pure fructose-1-phosphate; but further consideration led to a different conclusion. The proportion of fructose-1-phosphate in these products was probably not greater than in those of lower specific rotation and high aldose content, and whereas aldosephosphates could be quantitatively removed by oxidation, a method for the elimination of fructose-6-phosphate had still to be found. It was therefore decided to use crude bone extract for the partial hydrolysis of fructosediphosphate on a larger scale. Two such hydrolyses (Table I, Exps. 6 and 7) were carried out and yielded 7·5 g. and 8·4 g. respectively of barium hexosemonophosphates, equivalent to about 15 % of the total P of the diphosphate. From their analytical values it was judged that these products, like that of Exp. 5, contained approximately 30 % of aldosemonophosphates and 50 % of fructose-1-phosphate[1]. The residues from Exps. 1–7 contained considerable amounts of unchanged fructosediphosphate, which was separated from the inorganic phosphate by repeated precipitation of the acid barium salt; this was converted into sodium salt and partially hydrolysed with crude bone extract, whereby a further quantity (6·7 g.) of hexosemonophosphate of slightly lower aldose content was obtained.

Removal of aldosemonophosphates. 14 g. of the barium hexosemonophosphates were dissolved in 240 ml. of water and treated, at room temperature, with 1·5 ml. bromine; 70 ml. of a cold saturated solution of baryta (0·47 *N*) were added in 10 ml. portions at intervals during 20 minutes, with constant shaking; these quantities represented a 200 % excess of bromine and 50 % excess of baryta over the calculated amounts required for the oxidation of the aldosephosphate. After a further 5 minutes the solution was acidified with 12 ml. *N* HCl and the excess of bromine was removed by aeration for one hour. The p_H, which was then 4·2, was raised to 8·0 by addition of baryta and the precipitated barium phosphohexonate was filtered off; the fructosephosphates were recovered from the filtrate by precipitation with alcohol and were freed from phosphohexonate by repeated solution in small quantities of water and fractional precipitation with alcohol. The fraction finally precipitated between 18 and 66 % alcohol weighed 7·2 g. and had P 7·81 % and $[\alpha]_{5461} - 27°$. 6·7 g. of hexosemonophosphate obtained from recovered diphosphate were similarly treated and yielded 3·1 g. barium salt having P 7·63 % and $[\alpha]_{5461} - 23°$. The product obtained by Macleod and Robison [1933] had $[\alpha]_{5461} - 25·8°$.

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[1] The figures are best satisfied by assuming the presence of both glucose and mannose esters, but the exact composition cannot be calculated without more information on this point.

Separation of the fructosemonophosphates. An attempt was first made to separate the two fructosemonophosphates by fractional precipitation of the barium salts ($[\alpha]_{5461} - 27°$) from aqueous solutions by alcohol. Three fractions were obtained having $[\alpha]_{5461} - 28·7°$, $-27·1°$, $-24·2°$ respectively. The first, which amounted to 60 % of the total salt, contained the highest proportion of fructose-1-phosphate; but the degree of separation was small and it was thought well to try other methods before continuing this fractionation.

The next attempt was based on the possibility that in alkaline solutions fructose-6-phosphate might be converted into aldosephosphate (which could be oxidised with bromine) while fructose-1-phosphate remained unchanged. A number of tests were carried out with the mixed monophosphate (I value 3·5 %) obtained from Exp. 1 *a*; the results are briefly summarised below:

(*a*) In aqueous solution at p_H 8·4 and 28° no increase in the I value occurred in 11 days.

(*b*) In 0·01 *N* NaOH at 28° the I value slowly increased, reaching 6·6 % in 6 hours; but the H.J. value fell, in the same period, from 35 to 32 %; after 46 hours the I value had risen to 11 % and the H.J. value had fallen to 23 %, while 10 % of the total phosphate had been set free.

(*c*) In 0·05 *N* NaOH at 28° similar but more rapid changes occurred; in 1 hour the I value rose to 6·2 % and the H.J. value fell to 32 %; in 46 hours the H.J. value fell to 16 %, and 26 % of the phosphate was set free.

These results indicated that in 0·01 *N* and 0·05 *N* NaOH at 28° the fructose esters had undergone changes more profound than the simple conversion of ketose into aldose. In a final experiment 0·78 g. of the mixed monophosphates of low aldose content was treated with bromine in presence of a large excess of baryta (0·2 *N*) and the reaction was allowed to proceed for 100 minutes at room temperature. The amount of barium fructosephosphate recovered was only 0·12 g. and its specific rotation, −20°, was not very much higher than that of the original mixture. Destruction of both fructose esters had clearly taken place; the method was therefore abandoned as useless.

Recourse was now had to the fractional crystallisation of the brucine salts. It had been feared that this method might involve serious loss of the fructose-1-phosphate through hydrolysis during crystallisation or in the frequent interconversion of brucine and barium salts for analysis and further fractionation. Fortunately the brucine salts proved reasonably stable in aqueous and alcoholic solutions, while it was found possible to follow the course of their separation by measuring their specific rotations in aqueous solution; the loss in weight at 100° (Pregl micro-dryer) and P content of the salt were determined in each case. From time to time portions were converted into the barium salts and analysed; the amounts of hydrolysis occurring in 25 and 60 minutes in *N* HCl at 100° were also determined on a few mg. of the barium salts and furnished an indication of their purity.

The solution of the brucine salts in 80 % aqueous alcohol was prepared, as previously described [Robison and King, 1931], from 8·6 g. barium salts; it was cooled to −15° and, after crystallisation had begun, was left for a further 12 hours at 0°; weight of crystalline salt 21 g., representing 80 % of the total P. It was recrystallised three times by dissolving it in a small volume of 40 % alcohol at 30° and adding absolute alcohol to give 90 % concentration; weight after third recrystallisation 10·7 g. A small portion was converted into the barium salt for analysis; it had P 7·7 %, H.J. val. 28 %, I val. 1·5 %, $[\alpha]_{5461}-28°$; hydrolysis 88 % in 60 minutes. It was thus evident that very little separation had been achieved. The brucine salt was next dissolved in 35 ml. of 40 % alcohol and allowed to crystallise at 0°; four fractions, amounting to half the total salt, were removed and found to have an increased laevorotation, while the barium salt from one fraction had $[\alpha]_{5461}-31°$. Still better results were obtained by recrystallisation from water, in which the brucine salt was much less soluble than in dilute aqueous alcohol. Solution was effected at 40° and crystallisation allowed to proceed at room temperature. Successive fractions of the brucine salt had specific rotations ranging from −47 to −34°. All the fractions obtained in previous operations were therefore crystallised from water, and fractions of high rotatory power were combined and recrystallised. In this way the specific rotation of the brucine salt was gradually raised to −52°, that of the corresponding barium salt to −39° and the hydrolysis, in *N* HCl at 100°, to 98 % in 25 minutes and 100 % in 60 minutes.

The barium salt was purified by repeated solution in 10 parts of water and precipitation by alcohol. Traces of a sparingly soluble substance persisted and the salt was therefore again purified by fractional precipitation with alcohol; the main fraction obtained between 20 % and 70 % alcohol was filtered off, washed with absolute alcohol and dried in an evacuated desiccator. The analytical values for this salt are shown in Table III; the rates of hydrolysis of the free acid in 0·01 *M* aqueous solution (autolysis) and of the potassium salt in 0·1 *N* and *N* HCl at 100° are given in Table IV. It is seen that the value of *k* increases with the c_H of the solution; in this respect the behaviour of the ester agrees with that of fructosediphosphate and differs from the very abnormal behaviour of the

Table III. *Analyses of fructosephosphoric esters.*

Ester	Salt	Ba %	P %	Fructose (Selivanoff) %	Reducing power as glucose %		$[\alpha]_{5461}^{20°}$
					H.J.*	I	
Fructose-1-phosphate	Barium	34·5	7·82	24	26	0·9	−39·0 ($c=6·1$ %)
,,	Brucine	—	2·95	—	—	—	−52·1
,,	Free acid	—	—	—	—	—	−64·2 ($c=11·3$ %)
Fructose-1-6-diphosphate (used in present exps.)	Barium	—	10·42	8	12	0·7	+ 1·9°
Fructose-6-phosphate (from fermentation hexosemonophosphate)	,,	—	7·73	22	35	1·6	+ 2·3
Fructosemonophosphate (from hydrolysis of fructosediphosphate in *N* HCl at 100°)							
5 mins. hydrolysis†	,,	—	7·82	—	33	3·0	+ 2·6
30 mins. hydrolysis†	,,	—	7·79	—	33	2·0	− 5·1
30 mins. hydrolysis, 20 % alcohol fraction	,,	—	7·78	—	—	—	− 9·9

* Determined with addition of 0·5 ml. 0·5 *N* NaOH.

† These esters were hydrolysed to the same extent by *N* HCl at 100° in 25 and 60 min.

$C_6H_{10}O_4(PO_4Ba)_2$ requires P 10·16 %; $C_6H_{11}O_5PO_4Ba$ requires P 7·85 %; Ba 34·75 %; $C_6H_{11}O_5PO_4(C_{23}H_{26}O_4N_2)_2$ requires P 2·95 %.

Table IV. *Hydrolysis of fructose-1-phosphoric ester in acid solutions at 100°.*

Concentration of esters 0·01 *M*.

Time mins.	Autolysis		0·1 *N* HCl		*N* HCl			
					Found %		Calculated %	
	Found %	$k \times 10^3$	Found %	Calc. % for $k=9\times 10^3$	(*a*)	(*b*)	(*c*)	(*d*)
5	—	—	10·4	9·9	55·7	55·8	55·3	56·7
10	—	—	18·1	18·7	80·7	80·2	80·0	80·8
15	—	—	—	—	88·8	90·6	91·1	90·9
20	—	—	32·8	33·9	—	—	—	—
25	—	—	—	—	98·5	97·6	98·2	97·1
30	—	—	47·0	46·4	—	—	—	—
40	—	—	—	—	100·0	100·0	99·8	98·5
60	—	—	72·6	71·2	—	—	—	—
120	15·8	0·62	90·9	91·7	—	—	—	—
300	33·0	0·55	—	—	—	—	—	—
600	51·7	0·47	—	—	—	—	—	—

The values of *k* are calculated from the equation for a unimolecular reaction

$$k=\frac{1}{t_2-t_1}\log\frac{a-x_1}{a-x_2}.$$

For the hydrolysis in *N* HCl the values in columns (*a*) and (*b*) are those determined in two separate experiments. The values in columns (*c*) and (*d*) are calculated, (*c*) for 100 % of an ester having $k=70\times 10^{-3}$ and (*d*) for a mixture containing 98 % of an ester having $k=75\times 10^{-3}$ and 2 % of an ester having $k=4{\cdot}2\times 10^{-3}$. On the whole the observed values agree better with those in column (*c*) than with those in column (*d*)[1].

aldose-6-phosphates. The fall in the value of *k* during autolysis is probably due to the fall in c_H as phosphoric acid is set free.

The specific rotation of the barium salt is a little higher than the value previously calculated from the properties of the mixed fructosemonophosphates (−39° against −35°), while the value of *k* for hydrolysis in *N* HCl is a little lower (0·070 against 0·075). With such high rates of hydrolysis the possible errors of the micromethods employed may appreciably affect the calculated values of *k*, and the presence of 1 %, or even of 2 %, fructose-6-phosphate in our final product cannot be definitely ruled out. In Table IV the percentages of hydrolysis calculated for 100 % of an ester with $k=0{\cdot}070$ and of 98 % of an ester with $k=0{\cdot}075$ are shown side by side with the observed values. Another explanation is, however, suggested by the results noted in a later paragraph, which show that when hexosediphosphate is hydrolysed with *N* acid the fructosemonophosphate produced at first is dextrorotatory, but soon becomes laevorotatory as hydrolysis proceeds. The final value of *k* observed in this hydrolysis cannot, therefore, represent the value for the ester first produced, presumably fructose-6-phosphate, while the latter compound may conceivably be more dextrorotatory than has heretofore been supposed. The assumptions made for the second component of the fructosemonophosphate mixture were therefore not strictly correct. Further discussion of this interesting point will be postponed until the completion of experiments now in progress.

Reaction of fructose-1-phosphate with phenylhydrazine. 1·2 ml. of a solution containing 13 mg. of the free ester were treated with 0·2 ml. phenylhydrazine and 0·2 ml. glacial acetic acid. A portion of this solution left at 0° for 48 hours deposited no crystalline phenylhydrazone. The remainder was heated in a boiling water-bath for 20 minutes, cooled to 0° and filtered after some hours; weight of dry osazone, 13 mg. The mother-liquor, which gave no weighable precipitate on heating for a further 45 minutes, contained 1·36 mg. P, almost entirely as inorganic phosphate. The crude osazone contained only a trace (0·1 %) of P and, after recrystallisation, melted at the same temperature as glucosazone, alone and when mixed with the latter; the yield of glucosazone was equivalent to about 90 % of the original ester. Since only a very small percentage of this ester would have been hydrolysed in 20 minutes at the p_H of the solution, the liberation of phosphoric acid must be attributed to the oxidising action of the phenylhydrazine on the terminal group, to which the phosphoric acid is therefore most probably attached.

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[1] The sealed ampoules containing the acid solutions were immersed in a covered bath of vigorously boiling water. Variations in the temperature of the bath due to changes in barometric pressure might account for some of the observed differences in these rates. Hydrolyses (*a*) and (*b*) were carried out at 99·7° ($p=752$ mm.); a subsequent hydrolysis of the same ester, at 100·7° ($p=779$ mm.) gave *k*, 0·080.

Products of partial hydrolysis of fructosediphosphate by N H_2SO_4.

It had occasionally been noted that preparations of the Neuberg ester obtained in this laboratory by hydrolysis of fructosediphosphate with mineral acids at 100° were slightly laevorotatory; the possibility that this might be due to the presence of fructose-1-phosphate was investigated. A solution of the free diphosphoric ester in N H_2SO_4 was heated at 100° for 5 minutes and the barium hexosemonophosphate isolated in the usual way; the residual diphosphate was again heated in N H_2SO_4 for a further 25 minutes and the monophosphate again separated from the products. Both barium salts were purified by solution in water and reprecipitation by alcohol: their analyses are given in Table III. Unexpectedly, it was the second fraction, obtained after 30 minutes' heating, that proved to be laevorotatory (−5·1°), while the specific rotation of the 5-minute fraction (+2·6°) was close to that usually found for fructose-6-phosphate. Precipitation of the 30-minute product from aqueous solution by 20 % alcohol gave a fraction having $[\alpha]_{5461} - 9·9°$. These dextro- and laevorotatory salts were each hydrolysed to approximately the same extent by N HCl at 100° in 25 minutes and 60 minutes. The laevorotatory substance cannot, therefore, have been fructose-1-phosphate. The presence of a derivative of fructopyranose, containing the phosphoric acid group in position 3, 4 or 5, might indicate either that the fructosediphosphate is not, after all, homogeneous or that migration of the phosphoric acid group from position 6 occurs under the conditions of hydrolysis. If the latter explanation be correct it might also account for other puzzling results noted in the past, *e.g.* the failure to obtain pure glucose-6-phosphate, in more than small proportion, from trehalosemonophosphate after long-continued hydrolysis in N H_2SO_4. It would also mean that the final rates of hydrolysis of the hexosephosphoric esters in N HCl at 100° may not be those of the ester supposedly present. Further work on this question is in progress and will shortly be reported.

SUMMARY.

1. In continuation of the experiments of Macleod and Robison [1933] the hydrolysis of fructose-1:6-diphosphate by preparations of bone phosphatase has been further investigated. It has been shown that the products of hydrolysis by specially purified phosphatase are fructose-1-phosphate and fructose-6-phosphate in approximately equal proportions; aldo-derivatives are not formed.

2. The production of aldo-derivatives which was previously observed is due to the presence of a phosphohexokinase in crude bone extracts and in phosphatase

preparations obtained from these by addition of alcohol and ether. During further purification of the phosphatase this kinase is destroyed.

3. The phosphohexokinase is present also in marrow extracts and can be precipitated from these extracts by alcohol and ether; it is inactivated if the precipitate is allowed to remain for some hours in aqueous alcohol.

4. This phosphohexokinase is probably identical with the enzyme found by Lohmann in extracts of muscle, kidney, liver, brain and yeast. It converts fructose-6-phosphate and glucose-6-phosphate into equilibrium mixtures of aldo- and keto-esters. It has no apparent action on fructose-1-phosphate and none on fructose-1:6-diphosphate unless phosphatase is also present and first removes the phosphate group from position 1. It does not effect the conversion of free fructose into aldose.

5. Fructose-1-phosphate has been isolated in apparently pure condition from the products of hydrolysis of the diphosphate by fractional crystallisation of the brucine fructosemonophosphates from water.

The properties of the ester have been studied. The value of $[\alpha]_{5461}$ for the barium salt is $-39{\cdot}0°$, slightly higher than that calculated by Macleod and Robison [1933]; $[\alpha]_{5461}$ of the free ester is $-64{\cdot}2°$.

The rates of hydrolysis of the free ester in 0·01 M aqueous solution and in 0·1 N and N HCl at 100° have been determined. The values of k are for autolysis $0{\cdot}62 \times 10^{-3}$, for 0·1 N HCl 9×10^{-3}, for N HCl 70×10^{-3}.

6. When heated with phenylhydrazine in acetic acid solution for 20 minutes at 100° the ester is converted almost quantitatively into glucosazone. This confirms the previous conclusion that the phosphate group occupies position 1 in the fructose molecule. The ester is probably 1-phosphofructopyranose.

7. Other laevorotatory fructosemonophosphates have been obtained as the result of heating fructosediphosphoric ester with N H_2SO_4 at 100°. Experiments are in progress to discover the nature of these esters and to explain their formation.

REFERENCES.

Lohmann (1933). *Biochem. Z.* **262**, 137.

Macfarlane and Robison (1935). Unpublished results.

Macleod and Robison (1929). *Biochem. J.* **23**, 517.

—— —— (1933). *Biochem. J.* **27**, 286.

Martland and Robison (1929). *Biochem. J.* **23**, 237.

Neuberg (1918). *Biochem. Z.* **88**, 432.

Patwardhan and Robison (1935). Unpublished results.

Robison and King (1931). *Biochem. J.* **25**, 323.

—— and Morgan (1930). *Biochem. J.* **24**, 119.

—— and Rosenheim (1934). *Biochem. J.* **28**, 684.

CXVII. THE OXIDATIVE DISAPPEARANCE OF LACTIC ACID FROM BRAIN AND THE PASTEUR REACTION.

BY KENDAL CARTWRIGHT DIXON[1].

From the Biochemical Laboratory, Cambridge.

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MEASUREMENTS of the rate of oxidative lactic acid disappearance have been made by Holmes and Ashford [1930] and Ashford and Holmes [1931] in chopped brain, by Meiklejohn [1933] in minced tissue and by Meyerhof and Lohmann in brain slices [1926, 1]. Ashford and Dixon [1935] made some measurements of oxidative lactic acid disappearance and pointed out the small rate of this disappearance in relation to the rate of anaerobic lactic acid production. It seemed desirable to confirm this low value for rate of lactate disappearance in view of the doubt it throws on the generally accepted mechanism of the Pasteur reaction, namely, that oxygen removes the lactic acid which is continually formed as under anaerobic conditions. Further, it was necessary to establish if in reality more lactic acid disappears than can be accounted for by the oxygen uptake, as Ashford and Holmes [1931] have maintained.

In general the lactic acid oxidised is calculated (as Meyerhof did first for muscle) from the extra oxygen uptake caused by the addition of lactate. The justification for this method has been questioned in Ashford and Holmes's paper on the ground that sparing of the oxidation of intracellular substances may result from lactate addition and thus the oxygen uptake is really greater than that calculated. Ashford and Holmes concluded that sparing had not taken place.

Quastel and Wheatley [1932] however showed that lactate spared the oxidation of succinate. They also showed that the excess oxygen uptake caused by small addition of lactate to brain respiring without substrate would account for most of the lactate added if measured over a prolonged period of time. It seems evident however (as indeed they admit) that this only shows that complete oxidation may occur finally. Resynthesis in the Meyerhof sense might well have occurred at first, the resynthesised carbohydrate being later oxidised. Without continuous measurements of lactate disappearance this question could not be answered.

As the rate of respiration without substrate falls off markedly with time and that with added lactate only falls slightly, the difference between these values increases with time (this is seen from O_2 uptake curves in phosphate buffer [Holmes and Ashford, 1930; Ashford and Holmes, 1931] and is demonstrated below for bicarbonate buffer). It thus appeared that data as to the fate of the disappearing lactate could be obtained by studying the lactate disappearance and corresponding O_2 uptake over a period of several hours. If the accepted method of calculating lactate oxidation is correct (that is, that the difference between the lactate and no substrate respiratory values gives the lactate oxidation), then since this difference increases with time, the rate of disappearance of lactate should also increase. If on the other hand the oxidation of lactate suppresses the autorespiration, then the lactate will go on disappearing at the same rate. If this latter is really the case the lactate oxidised should be calculated not from the difference or excess O_2 uptake but from the total oxygen uptake.

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[1] Harold Fry Student, King's College.

METHODS.

All experiments were performed with slices of rabbit's brain cortex suspended in bicarbonate Ringer at 37°. Measurements of oxygen uptake and CO_2 production were made by the indirect (two vessel) method of Warburg. Sodium lactate made by neutralising *dl*-lactic acid with NaOH (with heating during the process to ensure hydrolysis of the lactide) was used so as to give a final concentration of 0·2 % lactic acid. Warburg measures aerobic lactic acid production by subtracting the O_2 uptake (Q_{O_2}) from the total CO_2 production in bicarbonate (Q_{SCO_2}). This method assumes a respiratory quotient of 1; if this is the case then that part of the total CO_2 production over and above an amount equal to the oxygen uptake is due to the formation of lactic acid and consequent decomposition of bicarbonate. Conversely, when lactic acid is disappearing the total CO_2 production is less than the oxygen uptake by an amount equal to that bound by the liberated cation of the lactate. This method has been described by Meyerhof and Lohmann [1926, 2]. The disappearance of lactate was thus measured by the value $Q_{O_2} - Q_{SCO_2}$[1] which gives the CO_2 absorbed owing to lactic acid disappearance and resulting liberation of base. This assumes R.Q. of 1 for the oxidation of lactic acid[2]. (The R.Q. for the autorespiration of brain slices is 1 [Dickens and Greville, 1933].) The values observed are essentially of the same order as those obtained chemically by Ashford and Holmes. The advantage of this method is that it readily gives the rates of lactic acid disappearance during the whole course of the experiment.

EXPERIMENTAL RESULTS AND DISCUSSION.

Table I shows rates of lactate disappearance ($-Q_L^{O_2}$), O_2 uptake (Q_{O_2}) and also total CO_2 production observed (Q_{SCO_2}) both with and without substrate. In

Table I.

		Lactate			No substrate		Glucose
Date	Remarks	Q_{O_2}	Q_{SCO_2}	$-Q_L^{O_2}$	Q_{O_2}	Q_{SCO_2}	$Q_L^{N_2}$
25. i. 35	2 parallel experiments	7·1	4·9	2·2	—	—	—
		7·9	5·7	2·2	—	—	—
30. i. 35	1st hour	7·4	5·4	2·0	4·25	3·3	—
	3rd hour	7·0	5·3	1·7	2·4	2·6	—
	4th hour	6·9	5·1	1·8	2·0	2·0	—
2. ii. 35	1st hour	7·9	4·7	3·2	3·9	2·1	14·3
	2nd hour	7·4	5·05	2·35	2·8	2·2	10·7
	3rd hour	7·1	5·0	2·1	2·3	2·4	9·7
	4th hour	6·7	4·4	2·3	1·9	2·0	—
15. ii. 35	1st hour	8·0	5·8	2·2	5·6	4·3	—
	2nd hour	6·6	4·7	1·9	3·1	2·6	—
	3rd hour	6·1	3·9	2·2	1·9	1·5	—
	4th and 5th hour	6·1	4·2	1·9	1·1	1·1	—
	6th hour	5·3	3·5	1·8	0·5	0·2	—
	Average	7·0	—	2·1	—	—	—

[1] $Q_{O_2} = \mu l$ O_2 uptake per mg. dry weight per hour. $Q_{SCO_2} = \mu l$ observed CO_2 output per mg. dry weight per hour. $-Q_{O_2}$, the rate of disappearance of lactic acid, is equal to $Q_{O_2} - Q_{SCO_2}$.

[2] If the R.Q. were less than unity, the disappearance of lactic acid would be still less; this would not alter the conclusions reached in this paper.

one experiment the anaerobic glycolysis from glucose was simultaneously measured and values of $Q_{\mathrm{L}}^{\mathrm{N}_2}$ are given.

It is first seen that the low values for lactate disappearance are amply confirmed, an average value of $-Q_{\mathrm{L}}^{\mathrm{O}_2}$ of 2·1 being obtained.

Secondly, although considerable fall in rate of autorespiration is shown with time, only a slight fall of lactate respiration rate is experienced. This is very strong evidence that lactate addition largely suppresses the autorespiration.

In the third place although the difference between the oxygen uptake with and without lactic acid increases with time, the rate of lactate disappearance maintains either a fairly constant level or even falls slightly, but does not rise as would be the case if the resynthesis theory were correct (see above).

We may conclude then that subtracting the O_2 uptake without substrate from that obtained with added lactate is not a correct method of obtaining a value for the lactic acid oxidised, and that this can be obtained with much more justification from the total oxygen uptake. The existence of resynthesis or unaccounted lactic acid disappearance in brain thus becomes very doubtful. It seems safe to assume that the lactic acid is completely oxidised. The average of the values in Table I ($Q_{\mathrm{O}_2}=7{\cdot}0$ and $-Q_{\mathrm{L}}^{\mathrm{O}_2}=2{\cdot}1$) is quite in harmony with this view.

Ashford and Holmes [1931] described experiments with washed chopped brain and also with chopped brain from animals killed in insulin convulsions. Under these conditions they obtained in some cases more lactate disappearing than could even be accounted for by total oxygen uptake. An experiment has been performed using brain cortex slices from a starved rabbit killed when in hypoglycaemic convulsions caused by an injection of 15 units of insulin 2½ hours previously. The results are shown in Table II.

Table II.

		Lactate			No substrate	
Date	Remarks	Q_{O_2}	Q_{sCO_2}	$-Q_{\mathrm{L}}^{\mathrm{O}_2}$	Q_{O_2}	Q_{sCO_2}
21. ii. 35	2nd half hour	7·8	5·3	2·5	4·55	3·7
	2nd hour	6·8	4·6	2·2	(1·7)	(−0·6)
	3rd hour	5·85	3·2	2·65	1·9	2·0
	4th hour	6·8	4·6	2·2	1·1	0·6
	5th and 6th hour	6·35	4·1	2·25	1·0	1·3
	7th hour	6·3	4·3	2·0	0·5	0·5

In this experiment the average Q_{O_2} in lactate (a mean of the values in the table) is 6·65, while the average lactate disappearance is given by $-Q_{\mathrm{L}}^{\mathrm{O}_2}=2{\cdot}3$. This result is nearer the theoretical 3 molecules of oxygen used for every lactic acid molecule oxidised than the values obtained with the normal brain slices. The small excess of the theoretical value of $Q_{\mathrm{O}_2}=6{\cdot}9$ over the observed $Q_{\mathrm{O}_2}=6{\cdot}65$ is almost certainly due to experimental error. In fact the agreement with the theoretical value is better than in the normal brain, where the Q_{O_2} of 7·0 instead of a calculated value of 6·3 was observed. It may well be that a very slight respiration of other substrate occurs in the normal brain and that this is removed by insulin with its attendant hypoglycaemia. The experiment shown in Table II shows the same general course of metabolism as in experiments recorded in Table I, that is, fairly steady disappearance of lactate, while rise in the difference between respiration in lactate and respiration without substrate occurs with increase in time. In this case then also the lactate oxidised should be calculated from the total oxygen uptake and not from the difference between the respiration in lactate and the autorespiration.

It seems clear that the hitherto much favoured theory of mechanism of the Pasteur reaction now requires, at any rate for brain, considerable modification. The rate of lactate disappearance in oxygen is no more than a quarter that obtained for lactic acid production in nitrogen (as is seen in Table I). This precludes the possibility that lactic acid is formed in oxygen as in anaerobic conditions and then removed. It might be said that the metabolism without substrate is still producing lactic acid and this is being re-oxidised to such an extent as to impede the disappearance of added lactate. This view, however, is rendered very unlikely for the following reasons. Firstly, it has been shown by Holmes and Holmes [1925; 1927], Ashford and Holmes [1931] and Holmes and Sherif [1932] that the anaerobic lactate formation without glucose is very small. Secondly, the metabolism without substrate seems to be largely suppressed in the presence of lactate. And, thirdly, the addition of potassium chloride does not cause aerobic glycolysis to appear in brain slices without substrate; this points to the absence of lactic acid formation and lactic acid-forming substance under these conditions.

The use of potassium chloride as an indicator for the presence of lactic acid-forming substances should here be briefly described. It has been shown [Ashford and Dixon, 1935] that addition of this substance causes appearance of marked aerobic glycolysis from glucose as well as increased oxygen uptake. It has since been shown that with fructose only increased oxygen uptake is observed and no aerobic lactic acid formation occurs. This was to be expected since Loebel [1925] and others since have shown that fructose gives hardly any anaerobic glycolysis. Lactate also gives increased oxygen uptake with potassium chloride. If, therefore, in the metabolism without substrate some lactic acid-forming substance were being used we should expect that the addition of potassium chloride would here cause aerobic lactic acid formation as with glucose. The following experiments with added potassium chloride in $M/10$ concentration negative this possibility: it is seen that without substrate there is no aerobic glycolysis, whereas with glucose and mannose which readily glycolyse under anaerobic conditions there is marked aerobic glycolysis.

Table III.

Date		No substrate	Glucose 0·2 %	Fructose 0·2 %	Mannose 0·2 %	Glucose 0·2 % Fructose 0·2 %	Glycogen 0·2 %
22. i. 35	$Q_{\mathrm{L}}^{\mathrm{O}_2}$*	0	14·5	0	—	13·7	—
19. ii. 35	$Q_{\mathrm{L}}^{\mathrm{O}_2}$*	0	10·7	—	11·0	—	0

* Rough direct method of Warburg [see Ashford and Dixon, 1935].

We may conclude then that in oxygen lactic acid is not formed at the same rate as in nitrogen and then reoxidised. Firstly, lactic acid does not disappear fast enough in oxygen for this to be the case. Secondly, if all the lactic acid formed in nitrogen were oxidised completely in oxygen the Q_{O_2} would be of the order of three times the $Q_{\mathrm{L}}^{\mathrm{N}_2}$—it is in fact equal to about half the $Q_{\mathrm{L}}^{\mathrm{N}_2}$. For this reason one would have to assume resynthesis of lactic acid or some other form of disappearance unaccounted for by oxygen uptake, to explain a high rate of disappearance. However, the experiments described here disprove the idea of resynthesis, since the lactic acid disappearing is completely oxidised, at any rate at the concentrations employed (0·2 %). It seemed possible that unaccounted disappearance might occur at higher concentrations (0·5 %) employed by Ashford and Holmes. However, an experiment performed using 0·5 % lactic acid has shown no unaccounted disappearance of lactic acid, as is seen in Table IV.

Table IV.

		Lactate (0·5 % lactic acid)			No substrate		Glucose
Date	Remarks	Q_{O_2}	Q_{SCO_2}	$-Q_L^{O_2}$	Q_{O_2}	Q_{SCO_2}	$Q_L^{N_2}$
13. iii. 35	1st hour	8·3	5·6	2·7	4·9	3.8	19·9
	2nd hour	7·4	4·8	2·6	3·5	2·8	17·5
	3rd hour	7·1	4·5	2·6	1·6	0 8	20·4
	4th hour	6·5	4·1	2·4	1·3	1·0	15·8
	5th hour	6·8	4·9	1·9	0·7	0·5	15·8
	Average	7·2	—	2·4	—	—	—

One must assume that in oxygen lactic acid is either not formed at all from glucose, or, if formed, is produced at a rate slow enough to be removed by complete oxidation. It thus appears that oxygen exerts its sparing effect on glycolysis at some point in the system prior to the formation of lactic acid.

SUMMARY.

1. Low values for rate of disappearance of lactate relative to rate of lactic acid formation in brain slices are confirmed, values averaging $-Q_L^{O_2}=2\cdot 1$ for lactate disappearance being obtained.

2. The rate of lactate disappearance is shown to remain fairly constant over prolonged periods of time. Only slight fall in respiration rate in lactate is observed, up to 35 % over 6 hours. Marked fall in respiration without substrate up to 90 % is simultaneously observed.

3. The bearing of these results on the question of the fate of lactic acid disappearing and on the site of action of oxygen in the Pasteur reaction is discussed. It is concluded that the lactic acid oxidised should be calculated from the total oxygen uptake and that complete oxidation occurs.

4. The use of potassium chloride for detection of possible lactic acid-forming substance under aerobic conditions is indicated.

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REFERENCES.

Ashford and Dixon (1935). *Biochem. J.* **29**, 157.
—— and Holmes, E. G. (1931). *Biochem. J.* **25**, 2028.
Dickens and Greville (1933). *Biochem. J.* **27**, 832.
Holmes, B. E. and Holmes, E. G. (1925). *Biochem. J.* **19**, 492.
—— —— (1927). *Biochem. J.* **21**, 412.
Holmes, E. G. and Ashford (1930). *Biochem. J.* **24**, 119.
—— and Sherif (1932). *Biochem. J.* **26**, 381.
Loebel (1925). *Biochem. Z.* **161**, 219.
Meiklejohn (1933). *Biochem. J.* **27**, 1310.
Meyerhof and Lohmann (1926, 1). *Biochem. Z.* **171**, 421.
—— —— (1926, 2). *Biochem. Z.* **171**, 381.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 725.

CXVIII. THE RELATION OF SODIUM TO CHLORINE IN THE MILK OF SHORTHORN AND GUERNSEY COWS.

BY TUDOR STANLEY GEORGE JONES
AND WILLIAM LEWIS DAVIES.

From the National Institute for Research in Dairying, Shinfield.

(*Received March 2nd, 1935.*)

THE determination of the distribution of the cations in milk has, until recent years, been rendered difficult by the lack of a rapid and convenient method for the determination of sodium. The description by Barber and Kolthoff [1928] of an accurate gravimetric method by which micro-quantities can be determined by a macro-technique was followed by the description of a colorimetric method by McCance and Shipp [1931]. Earlier methods for the determination of sodium in milk have been described by Blanchetière [1923] and Barthe and Dufilho [1926; 1928, 1]. The method for the determination of sodium described in this paper consists of the application of the gravimetric method of Barber and Kolthoff to milk. Owing to the uncertainty of the temperature at which sodium is volatilised as chloride and the prolonged time necessary for complete ashing at the maximum allowable temperature, it was decided to adopt the method of wet ashing with nitric acid.

Few values for the sodium content of cow's milk have appeared in the literature and these are usually given as mean values together with the ranges in which they occur. Little attempt has been made to correlate the sodium and chloride values except to state that the chloride in milk is more than equivalent to the sodium and that the excess is probably combined with calcium. Indeed it has been stated [Barthe and Dufilho, 1927] that the sodium content of the milk of healthy cows is never greater than 50 mg. per 100 ml. and that the Na : Cl ratio is never higher than 0·339. The idea however is presented that the sodium content of cow's milk increases as the period of lactation advances [Barthe and Dufilho, 1928, 2].

The milk of 20 cows of the Shorthorn and Guernsey breeds has been examined in an attempt to correlate the sodium and chloride contents of their milk and their stages of lactation. The results of duplicate, and in some cases triplicate, determinations are given in Table I together with the length of time during which the cows had been in lactation. The values for sodium and chloride have ranged from 39·2 to 139·2 and 70 to 193 mg. per 100 ml. respectively, with mean values of 76·8 and 113·4 mg. per 100 ml. respectively. The calculation of the correlation coefficient for the collected data has resulted in the high value of 0·91 and the straight line representing this correlation, calculated by the method of least squares, is represented by the equation

$$Cl = 1{\cdot}24\,Na + 18{\cdot}09$$

where Cl and Na are expressed in mg. per 100 ml. This is the straight line drawn in the graph (Fig. 1). This correlation is given independently of the breed of the cow, no significant difference having been found between the milks of the two breeds investigated.

When expressed in milli-equivalents, the relation takes the form

$$\mathrm{Cl} = 0{\cdot}805\,\mathrm{Na} + 0{\cdot}51$$

from which it is evident that chloride is always in excess. This holds for values of Cl from 70 to 193 mg. per 100 ml., the higher value being well into the region of abnormality preceding "drying off". It is not certain that the equation holds for values of chloride below 70 mg. per 100 ml. (or sodium below 39·2 mg. per 100 ml.) since samples of cow's milk of lower chloride content are rarely encountered. The Na: Cl ratio is evidently not a fixed quantity for milk, but is a function of the value of either element, so the statement of Barthe and Dufilho [1927] is incorrect. Indeed, in no cases were values as low as 0·339 found for this ratio in the milk of the cows of our herd, which is to be regarded as typical of English herds of their breeds. The value given for the mean of the

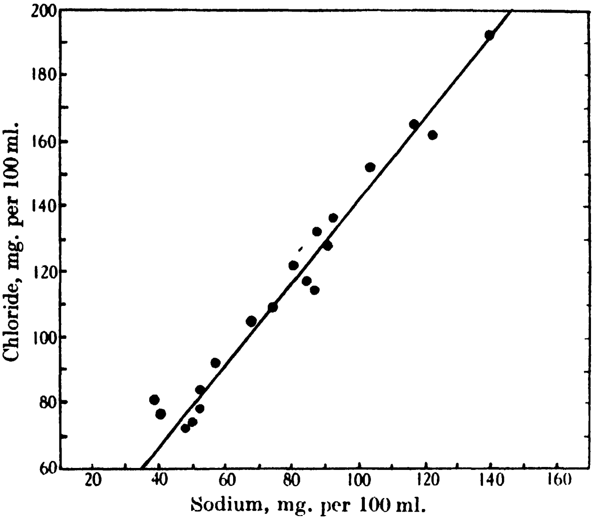

Fig. 1. Relation of sodium to chloride. $\mathrm{Cl} = 1{\cdot}24\,\mathrm{Na} + 18{\cdot}09$; $r = 0{\cdot}91$.

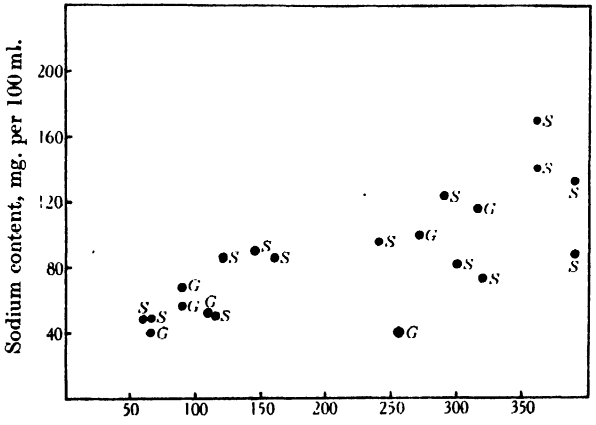

Fig. 2. Sodium content and duration of lactation.

chloride contents of the milks examined is somewhat higher than the value found for over a thousand samples [Davies, 1935] which was 100 mg. per 100 ml. The latter corresponds to a sodium value of 66·0 mg. per 100 ml. which can be regarded as the mean sodium content for cow's milk.

As is well known, the chloride content of milk increases with advance in the period of lactation. Consequently, the value for the sodium content of milk must increase. Fig. 2 shows the scatter diagram for sodium against the period of lactation in days. Again it is evident that there is no significant difference between Shorthorn and Guernsey types. There appears to be a general upward trend but the number of determinations is not large enough for statistical treatment.

METHODS.

The milk samples used were taken from individual cows either at the a.m. or p.m. milking and represent the whole of the milk at that particular milking (fore milk being rejected).

Chloride was determined by the method of Davies [1932]. Sodium was determined by the triple acetate gravimetric method as follows:

Reagents. (1) Concentrated nitric acid (sp. gr. 1·4).

(2) Alcoholic zinc acetate with zinc hydroxide. This is prepared from sodium-free zinc sulphate according to the method of McCance and Shipp [1931].

(3) Zinc uranyl acetate solution: (*a*) 10 g. of uranyl acetate (A.R.) are dissolved in 50 ml. of boiling water containing 2 ml. of glacial acetic acid; (*b*) 30 g. of zinc acetate are dissolved in 50 ml. of boiling water containing 1 ml. of glacial acetic acid. (*a*) and (*b*) are mixed while boiling, re-boiled for a few minutes, cooled, allowed to stand overnight and filtered. This solution is always kept saturated at working temperature with sodium zinc uranyl acetate, prepared by mixing some of the reagent with an alcoholic solution of sodium chloride, and filtered before use.

(4) 95 % alcohol saturated with the triple acetate. The alcohol is kept in contact with the triple acetate at working temperature and filtered before use.

Procedure. 25 ml. (or if the milk is known to contain less than 80 mg. Na per 100 ml., 50 ml.) of milk are measured into a 150 ml. silica flask, 10 ml. of concentrated nitric acid are added, and the mixture is heated on a sand-bath. When the solution has been almost reduced to dryness the mass chars and the carbon ignites and burns in the nitric fumes. By the cautious addition of nitric acid it is possible to obtain a white ash in a few minutes. This ash is dissolved in a small amount of dilute nitric acid and transferred with the aid of 10 ml. of water to a 25 ml. graduated flask. For removal of phosphate 15 ml. of the zinc acetate-zinc hydroxide reagent are added, mixed with the solution and allowed to stand 24 hours. The solution is made up to the mark and filtered. 2 ml. portions of the filtrate are measured into 10 ml. of the zinc uranyl acetate reagent contained in a 10 G 4 Jena filter of 15 ml. capacity which is stoppered at the bottom in the manner described by Peters and Van Slyke [1932]. After vigorous stirring by means of a short, thin rod, the latter is washed down by two successive portions of 1 ml. of the reagent and the filter is covered and set aside for 1 hour. After removal of the stopper, the fluid contents are sucked through by means of the pump and the precipitate is washed five times with 2 ml. portions of alcohol saturated with the triple acetate and twice with 5 ml. portions of ether. The outside of the filter is then wiped dry, the filter is placed in a desiccator for 1 hour and weighed, an ordinary analytical balance being sufficiently accurate. The precipitate weighs 66·9 times its content of sodium, so that using 25 ml. of milk, and 2 ml. of filtrate, the mg. Na per 100 ml. milk are

$$\frac{100 \times \text{mass of precipitate (mg.)}}{2 \times 66{\cdot}9}.$$

The agreement between triplicates is usually well within 1 % and often identical values are obtained.

Excess of potassium interferes with the determination, but this element does not occur in milk to such an extent that special precautions have to be taken as is the case with certain urines, feeding stuffs *etc.*

Table I.

Cow No.	Guernsey (G) or Shorthorn (S)	Days in milk	Milk Cl mg./100 ml.	Milk Na mg./100 ml.
1	G	255	81	39·2
2	G	88	92	56·3
3	G	87	105	68·0
4	S	118	117	86·0
5	S	239	140	97·2
6	G	314	165	116·5
7	S	351	193	139·2
8	S	324	109	73·7
9	S	297	122	82·2
10	S	64	70	48·8
11	S	145	128	90·7
12	S	160	116	85·8
13	S	288	162	121·6
14	G	66	77	39·9
15	S	387	132	87·4
16	G	268	152	100·8
17	G	109	84	52·1
18	S	116	74	50·0
19	G	108	78	52·1
20	S	64	70	48·2

SUMMARY.

1. A detailed account of the determination of sodium in cow's milk is given.

2. In the milk of 20 cows the average values of Cl and Na were 113·4 and 76·8 mg. per 100 ml. respectively.

3. The relation between chloride and sodium in milk of Shorthorn and Guernsey cows is given by the equation

$$\text{Cl} = 1{\cdot}24\ \text{Na} + 18{\cdot}09$$

where Cl and Na are expressed in mg. per 100 ml.

REFERENCES.

Barber and Kolthoff (1928). *J. Amer. Chem. Soc.* **50**, 1625.
Barthe and Dufilho (1926). *Compt. Rend. Acad. Sci.* **182**, 1470.
—— —— (1927). *Ann. Falsif.* **20**, 88.
—— —— (1928, 1). *Ann. Falsif.* **21**, 578.
—— —— (1928, 2). *Le lait*, **8**, 97.
Blanchetière (1923). *Bull. Soc. Chim.* **33**, 807.
Davies (1932). *Analyst*, **57**, No. 671, 79.
—— (1935). Unpublished work.
McCance and Shipp (1931). *Biochem. J.* **28**, 449.
Peters and Van Slyke (1932). Quantitative clinical chemistry, **2**, 732.
(Baillière, Tindall and Cox, London.)

OBITUARY NOTICE.

JOHN ALEXANDER MILROY.

(1871–1934.)

JOHN ALEXANDER MILROY, one of the Original Members of the Biochemical Society, died at Belfast after a brief illness on September 19, 1934, having just passed his sixty-third birthday. Born in Wigtownshire, he received his school and university education in Edinburgh, obtaining in 1893 the degree of M.A., in 1896 that of M.B. and in 1902 that of M.D. While still a medical undergraduate he spent a winter in Salkowski's laboratory in Berlin, and at a later date he was for a time a student under Emil Fischer. After graduation he acted as Demonstrator of Physiology first in Owens College, Manchester, and then in Yorkshire College, Leeds. In 1902 he accompanied his elder brother, Prof. T. H. Milroy, to Queen's University, Belfast, where the latter had just been appointed to the Chair of Physiology. At Belfast he held at first the post of Lecturer in Physiology, but in 1909 was appointed Lecturer, and in 1922 Reader, in Biochemistry. In 1924 he was promoted to the newly-founded J. C. White Professorship of Biochemistry. It had been his intention to terminate his tenure of the Chair after one more year of service, and to retire from active academic work at the same time as his brother in 1935. An association, which had endured already for thirty-two years, would thus have found an appropriate and felicitous conclusion.

In 1902 Milroy submitted to the University of Edinburgh a thesis for which he was awarded the degree of M.D. with the added distinction of a gold medal. The title of this thesis—"A contribution to the knowledge of the products of the reduction of haematin"—indicates his early interest in a field, to which in succeeding years he made a variety of important contributions. He had already, as a matter of fact, presented to the Physiological Society (*J. Physiol.*, 1901) a preliminary account of the matters dealt with in his thesis, and in 1904 he communicated through the same channel a paper on "Products of the distillation of haematin with zinc dust" and another on "Reduced acid haematin and some of its derivatives." In 1909 he described a remarkably stable derivative of haemochromogen, in which iron had been largely replaced by nickel, and also reported a careful measurement of the specific CO-capacity of reduced acid haematin (*J. Physiol.*, 1909). The last paper of this series (*Biochem. J.*, 1918) describes the preparation, the absorption spectra and the properties of a number of metallic compounds of haematoporphyrin, some of them previously unknown. Notable among these was the stannous compound, which was found to be remarkably stable and to show a spectrum of singular intensity. Upon this observation Milroy based a new test for blood pigment, exceeding in delicacy any other known test of specific character.

The group of papers just cited embodies in itself a substantial contribution to Biochemistry. Milroy's other publications show that he took a lively and productive interest in many widely different topics. They include papers on albumosuria (*J. Path.*, 1901), on the staining of protein crystals (*Proc. Scot. Microscop. Soc.*, 1902), on the estimation of urea (*Biochem. J.*, 1913), blood sugar (*Biochem. J.*, 1925) and blood fat (*Biochem. J.*, 1928), and on sublimation *in vacuo* as a method of separating certain chemical constituents of the body (with S. Andrews, *J. Physiol.*, 1930). Latterly he had turned his attention to the pentose compounds of the animal organism; and in his last paper, planned as the first of a series, he dealt (again in collaboration with S. Andrews) with the micro-determination of free and combined pentoses (*Biochem. J.*, 1933).

John Milroy was joint author with his brother Thomas of a well-known text-book on Practical Physiological Chemistry, first published in 1904. A revision and extension of this book, leading to the issue in 1921 of a third greatly enlarged edition, was to a preponderating extent the individual work of the younger brother. The task was one which he performed in the spirit rather of the investigator than of the compiler; and the book incorporates much that must be credited to him as original work. The improvement of practical instruction in his Department was indeed an object in the pursuit of which Milroy spared himself no pains. The thoroughness of his methods was appreciated even by junior students, still more by the many graduates, both in Science and Medicine, who attended his advanced course.

Those fortunate enough to have known John Milroy are aware that his gifts of intellect were not more conspicuous than the charm of his personality and the mingled strength and sweetness of his character. The vivid smile that lit his face in conversation reflected a mind and a disposition at once eager and ingenuous. His modesty, his sociability, his kindly courtesy endeared him to all, young or old, with whom he came in contact. The interests of others always claimed his attention more readily than his own, and to any colleague who called upon him for information or advice he offered his time and his knowledge with prodigal liberality. Happy in his daily work, happy in his many friendships, he was happiest of all in the family life which he shared with his two brothers and two sisters—all, like himself, unmarried. All those—and they are many—who have been admitted to that closely-knit but hospitable household will understand, and share, the sorrow of its surviving members.

A. HUNTER.

CXIX. THE RÔLE OF THE PLACENTA IN THE FAT METABOLISM OF THE RABBIT FOETUS.

BY ELDON MATHEWS BOYD.

From the Department of Pharmacology, Queen's University, Kingston, Ontario, Canada.

(*Received January 8th, 1935.*)

IN recent years, the author has been interested in the study of fat metabolism during the reproductive cycle. The results of previous investigations leading to the present contribution may be briefly indicated. The preliminary step consisted in the elaboration of a method whereby all known lipoids could be simultaneously estimated in micro-amounts, in the same extract and using the same analytical principle—isolation followed by oxidation [Boyd, 1933, 1]. This differential micro-analysis was then applied to a study of lipoids in the blood of pregnant women in whom it was shown [Boyd, 1934, 1] that the concentration of lipoids is increased in plasma but not in the red blood cells and that the increase in plasma is different in regard to time and extent for each lipoid. The white blood cells were noted to contain smaller amounts of fatty substances in pregnant [Boyd, 1934, 2] than in non-pregnant [Boyd, 1933, 2] women but after parturition there occurred a remarkable increase of several hundredfold in the phospholipin, free cholesterol and neutral fat of these cells. Experiments which have not been published to date confirmed the observations of Baumann and Holly [1926] and others that in rabbits the blood lipoids are decreased rather than increased during pregnancy. In the ovary of pregnant and pseudopregnant rabbits, certain changes were encountered in lipoid composition which were interpreted as signifying that the physiological activity of this organ in this species increases up to the mid-point of pregnancy and retrogresses during the latter half [Boyd, 1935, 1].

Two possible effects of the lipaemia of pregnancy in women have so far been investigated. It has been shown that normal lactation following the termination of pregnancy is accompanied by a decline in the value of all plasma lipoids but that if lactation is prevented by drying up the breasts the lipaemia persists or may be augmented [Boyd, 1935, 2]. This was interpreted as signifying that the lipaemia assists in the production of milk fat or that the prevention of lactation causes some further maladjustment in fat metabolism. The second effect investigated was the possibility that the lipaemia may act in the nature of an increased pressure head forcing lipoids into the umbilical circulation through the placenta and on to the foetus where they are utilised in the building up of the developing organs. In this connection, it was found that the human placenta at full term pregnancy adds considerable amounts of lipoids to umbilical blood from which they are absorbed by the growing offspring [Boyd and Wilson, 1935]. The average large, well-nourished infant was recorded to absorb in the neighbourhood of 50 g. of lipoids per day from umbilical blood, 40 g. of these being phospholipin and the remainder equally divided between free cholesterol and cholesterol esters; neutral fat was occasionally absorbed and occasionally given up by the foetus.

From these results it was concluded that the placenta plays an important part in the transfer of lipoids from the mother to the foetus *in utero*. Two possible explanations may be offered regarding the manner in which this exchange takes place. First, lipoids in the maternal blood-stream may simply be passed across the placental membrane to the umbilical blood. In referring to this process, the term permeability has been purposely avoided since the manner in which lipoids are transferred across animal membranes is at present obscure; it may be through the intermediate formation of semi-soluble phospholipin although even this is disputed. Secondly, the placenta may act in the nature of a secreting gland, taking up lipoids from the maternal blood-stream and passing them on, with or without structural modification, to the foetal circulation. The absence of glandular tissue from the placenta in no way militates against this conception, since apparently all tissues of the body are capable of taking up and discharging lipoids of the blood. If the tissues of the placenta are actively engaged in the production or elaboration of lipoids for the foetus, then it appears that any alteration in the lipoid composition of the foetus at the various stages of pregnancy may be accompanied by corresponding changes in the lipoid composition of the placenta. The present investigation was concerned with the development of this postulate.

Rabbits were used in this investigation because the placenta in this species is functionally similar to that in man, *i.e.* it is of the haemo-chorial type, and hence results obtained with this animal may be more readily applied to and compared with our previous findings in the human placenta [Boyd and Wilson, 1935]. The procedure adopted was to select female rabbits at different periods during pregnancy, open the abdomen and uterus, take out a foetus with its corresponding placenta and then measure the lipoid content of each. When a sufficient number of such experiments had been done, it was possible to plot the lipoid composition of the foetus against the lipoid composition of the placenta at the various stages of pregnancy, noting whether or not there were any indications of variations in the fat metabolism of the placenta during gestation.

EXPERIMENTAL PROCEDURE.

Foetuses and placentas were obtained from the uteri of gravid rabbits at various days during pregnancy from the 14th day to the 32nd day or term. No determinations were made earlier than the 14th day because the foetus before this date is too small for a complete lipoid micro-analysis. The structures were kept in pairs so that the lipoid composition of the foetus could be compared with that of the placenta to which it had been attached while in the uterus. After removal from the uterus, the foetus and placenta were separately weighed and ground up to a fine mud in a mortar using rough quarry sand which had been de-carbonated with hydrochloric acid, washed with water and extracted with alcohol and was of a uniform size between 18 and 20 mesh. The mixture of sand and tissues was transferred quantitatively to a flask and shaken with at least 50 volumes (*v/w*) of a mixture of alcohol and ether, 3 : 1, both redistilled and the ether peroxide-free. The mortar was washed out several times with small amounts of this solvent and the tissue-sand, which tended to cake when the proteins were precipitated, broken up with a clean spatula. The contents of the flask were then brought to boiling on a steam-bath for a few minutes, thus completing the extraction of lipoids. After cooling to room temperature, the extracted fatty substances were filtered off through alcohol-extracted filter-paper into a volumetric flask, the residue was washed with repeated portions of alcohol-ether, and the combined filtrate made up to volume. Foetuses weighing over 10 g. were cut into fine pieces and continuously extracted with 10 to 15 volumes of alcohol-ether, for 10 to 15 hours in Bloor percolator-extractors. Both of these methods were found to give complete extraction and the choice was purely for convenience in adjusting the final volume of the extracts, the first method requiring large amounts of solvent and a short extraction time and the Bloor method using smaller amounts of solvent but a longer extraction time.

In each extract the lipoids determined experimentally were total fatty acids, total cholesterol, free cholesterol and phospholipins, using the Bloor oxidative micro-technique as modified by Boyd [1933, 1]. From these values determined experimentally were calculated the concentrations of the various lipoids present. Thus phospholipin and free cholesterol were obtained directly, ester cholesterol calculated by subtracting the value of free cholesterol from total cholesterol, and neutral fat by subtracting from the total fatty acids the fatty acids of phospholipin and cholesterol ester. These residual fatty acids, which were taken to represent neutral fat fatty acids and calculated to comprise 95 % of the neutral fat fraction, are probably present in the tissues mostly in the form of glycerol esters but may have other combinations and some may be present as free fatty acid. In view of this probability and of the absence of definite knowledge concerning their nature, they have been referred to collectively as neutral fat.

EXPERIMENTAL RESULTS.

The concentrations of the four main groups of lipoids, namely phospholipins, free cholesterol, ester cholesterol and neutral fat, in the rabbit foetus and placenta during the latter two-thirds of pregnancy are depicted respectively in Figs. 1, 2, 3 and 4. Curves for total lipoid, total fatty acid, total cholesterol and the other lipoids have not been given because they are not essential to the interpretation of the data and because they may be calculated approximately from the information given. The ordinates and abscissae in all figures are in the same units. The ordinate represents mg. of lipoid per 100 g. tissue, wet weight. The abscissa represents the weight of the foetus in g. In order to demonstrate clearly the variations in the smaller foetuses, the progression of the abscissa was made geometric rather than arithmetic. This should be remembered when interpreting the slope of the curves. The weight of the foetuses has been used for the abscissa rather than the day of gestation because the lipoid composition was found to vary with the weight of the foetus rather than with the duration of pregnancy. This was particularly apparent when three or four foetuses were analysed from a single uterus, especially in the earlier stages of gestation. To obtain the approximate duration of pregnancy in each curve, the following figures may be given as guides: at 14 days, average weight of foetus about 0·25 g.; at 17 days, 1·0 g.; at 24 days, 12 g.; at 31 days, 40 g.

A. *Phospholipins.*

The concentration of phospholipins in the foetus (Fig. 1) increased in the earliest embryos studied and in every embryo throughout pregnancy whenever the weight of the foetus increased, the most rapid increase being around the 14th day of gestation. As the weight of the foetus rose from 0·2 to 0·4 g., the phospholipin content rose from 122 to 513 mg. per 100 g. From then until the termination of pregnancy, phospholipin continued to rise in value but at a much smaller rate. At the termination of pregnancy, phospholipin composed on the average 1 % of the weight of the foetus.

While the phospholipin content of the foetus was steadily increasing, the phospholipin content of the placenta was found to exhibit two phases. As the foetus increased in weight from 0·2 to 2 g., the concentration of phospholipin in the placenta fell from over 1500 mg. per 100 g. to about 1000 mg. per 100 g. From then on to term, phospholipin rose to and remained at a plateau level of around 1500 mg. per 100 g., with considerable variation both high and low in different placentas. The appearance of this double phase became obvious only after examining a number of placentas, particularly those in the earlier stages of gestation. It is not shown in the results of previous investigators on other types of placentas; most of this previous work reports only one or two stages, but Watanabe [1923] obtained a "step down" type of curve for the human placenta, that is a high plateau in the first trimester, a drop to a lower plateau in the second trimester and then the lowest level of all in the last trimester. The only previous report on the placenta of the rabbit gives 600 mg. per 100 g. as the value for phospholipin on the 29th day of gestation [Baumann and Holly, 1926].

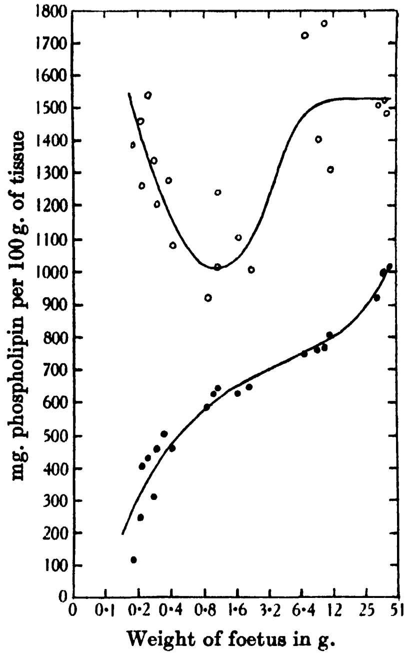

Fig. 1.

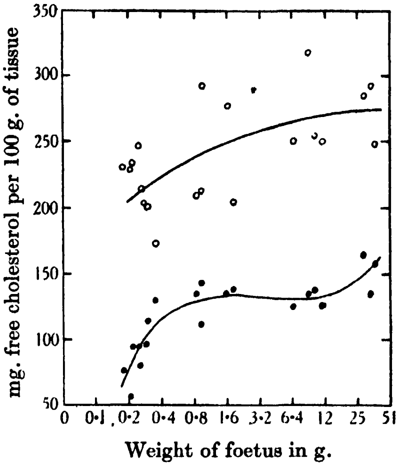

Fig. 2.

Fig. 1. Changes in the percentage of phospholipin in the foetus and in the placenta of the rabbit during intra-uterine growth. o—o placenta; •—• foetus.

Fig. 2. Changes in the percentage of free cholesterol in the foetus and in the placenta of the rabbit during intra-uterine growth. o—o placenta; •—• foetus.

The results suggest that the initial fall in the level of placental phospholipin is due to removal of phospholipin either to certain maternal organs such as the mammary glands, which were found by Baumann and Holly [1926] to have an infiltration of phospholipin during pregnancy, or to the foetus in which there is a very rapid increase in phospholipin as shown above. Since the weight of the placenta increases during pregnancy more rapidly than the concentration of phospholipin falls, there is actually a slight increase in the total amount of phospholipin segregated in this organ. Hence the increase in the phospholipin of the foetus cannot be due to a simple transfer of a certain number of mg. of phospholipin from the placenta. The phospholipin content of a tissue is a dynamic value, being increased by absorption of lipoid from the blood and decreased by oxidation, hydrolysis, and removal by the blood. Hence the placenta of early pregnancy, or rather the early part of the second half of pregnancy, may be giving up to the foetus or to some other organ more phospholipin than it actually requires to satisfy its own needs.

B. *Free cholesterol.*

Changes in the concentration of free cholesterol (Fig. 2) in the foetus during pregnancy closely paralleled the changes in phospholipin. This was not unexpected since the two lipoids appear to be closely related in vital economy, the one being antagonistic to the other in many physiological reactions, for example, haemolysis of the red blood cells. In the foetus, free cholesterol rose from a value of 58 mg. per 100 g. in a 0·2 g. embryo to 167 mg. per 100 g. in a 34 g. embryo almost at term. Again the increase in the foetus was more marked in the earlier cases studied.

The placenta exhibited considerable variation in regard to its free cholesterol with a tendency toward increasing amounts of the lipoid as gestation proceeded. The curve as represented in Fig. 2 was considered best adapted to the various points. The relationship to phospholipin may be shown more clearly if the first eight points for free cholesterol in the placenta are united by a straight line progressing downward followed by a rise in the latter part of pregnancy as in the case of phospholipin. In such a case, most of the discussion which was applied to phospholipin may also be applied to free cholesterol. Be this as it may, the results clearly indicate that cholesterol metabolism of the placenta varies according to the duration of pregnancy.

C. *Ester cholesterol.*

This lipoid underwent a remarkable change in the rabbit placenta during pregnancy. As shown in Fig. 3, it rose from a value of 139 mg. per 100 g. on the 14th day of gestation to almost 500 mg. per 100 g. between the 16th and 22nd days of pregnancy. After that, the concentration of placental ester cholesterol fell and at the end of pregnancy the value was similar to that found in the 14-day animals. During this time, small and comparatively insignificant amounts of ester cholesterol were present in the foetus, the values varying between 0 and 98 mg. per 100 g. but showing no definite trends.

Variations in the concentration of placental ester cholesterol are in many respects the opposite of those for phospholipin and free cholesterol, particularly phospholipin. Thus, while the values for phospholipin fell in the placenta between 0·2 and 10 g. foetuses, the ester cholesterol rose. Ester cholesterol in tissues is bound with fatty acids and the results suggest that the decrease in phospholipin may have been due to a liberation of fatty acids which were then combined with cholesterol to produce cholesterol esters. The concentration of total cholesterol also increased in this period, so that there must have been a considerable infiltration or synthesis of cholesterol.

It is worthy of note that the placenta of the rabbit was found to contain more cholesterol esters than are usually found in tissues. On the average, about one half of the total cholesterol was present as ester; in adult tissues, ester cholesterol rarely comprises more than 10 % of the total cholesterol. Since cholesterol esters appear to act as transporters of fatty acids [Bloor, 1924;

Schaible, 1932; Ling, 1931], there may be some significance in the high cholesterol ester content of the placenta in relation to the postulated transport of fatty acids to the foetus.

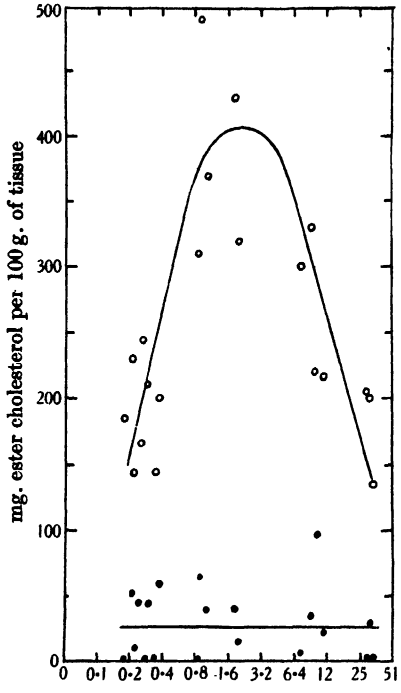

Fig. 3.

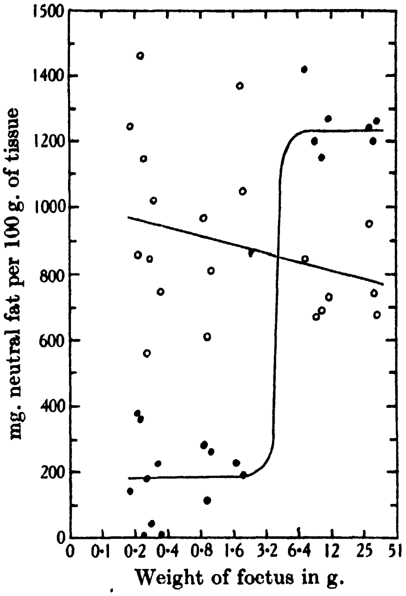

Fig. 4.

Fig. 3. Changes in the percentage of ester cholesterol in the foetus and in the placenta of the rabbit during intra-uterine growth. o—o placenta; •—• foetus.

Fig. 4. Changes in the percentage of neutral fat in the foetus and in the placenta of the rabbit during intra-uterine growth. o—o placenta; •—• foetus.

D. *Neutral fat.*

Comparatively little is known about the physiology of neutral fat apart from the fact that it is the chief storage form of fat in the body. Analyses for neutral fat in the rabbit foetuses, Fig. 4, confirmed the well-known clinical observation that premature and abortive embryos have a thin, emaciated appearance in contrast to the plump, well-rounded out appearance of full-term foetuses. These latter embryos owe their appearance to the presence of subcutaneous fat which is mostly neutral fat. In the foetuses of rabbits studied, neutral fat increased over 600 % between the 14th and last days of pregnancy, the rise taking place about the 20th day.

The neutral fat of the placenta varied within a wide range of values, especially in the earlier cases studied wherein figures were obtained between 560 and 1470 mg. per 100 g. Although the differences between placentas were also wide at subsequent periods of pregnancy, in general the values tended to fall somewhat. The total loss of neutral fat to the placenta was less than the total gain in neutral fat of the foetus.

DISCUSSION.

The present investigation offers certain further information concerning the relationship between the lipoid composition of the foetus and the activity of the placenta. It has been shown that the embryo of the rabbit acquires increased concentrations of phospholipin and free cholesterol as it grows in the uterus. There was no relation found between growth and the cholesterol ester content of the foetus, but about 10 days before the end of intra-uterine development the neutral or glyceride fat of the foetus increased sixfold. At birth the foetus of the rabbit contained a much higher percentage of all lipoids except cholesterol esters than in the early stages of growth within the mother.

The rates of increase in the concentrations of phospholipin and free cholesterol of the foetus were greatest up to about the 16th day or middle of pregnancy. Then ensued slower rates of increase until the last week of gestation when again the percentage of these lipoids rapidly increased. Phospholipin and free cholesterol, especially phospholipin, have been shown in recent years to increase in concentration in a tissue or organ whenever that tissue or organ becomes physiologically active [Bloor and Snider, 1934; Boyd, 1935, 1]. If it may be assumed that the rate of increase in physiological activity varies as the rate of increase in phospholipin, then the embryo of the rabbit may be said to exhibit two stages of marked activity, the first up to the 16th day and the second in the last week of intra-uterine life. The activity of the embryo in the first half of pregnancy is probably referable chiefly to growth. In the last week of gestation, however, the growth rate is falling off and it seems from the data obtained that activity of the embryo at this time is due to increased activity of fat metabolism, resulting in the deposition of large amounts of neutral or storage fat.

This stimulus to foetal fat metabolism in the last days of intra-uterine development may be shown to be accompanied simultaneously by an increase in the physiological activity of the placenta. As previously stated, physiological activity is characterised by increasing percentages of phospholipin and free cholesterol. On the other hand, degeneration, retrogression or inactivity results in a decrease in phospholipin and an increase in cholesterol esters [Bloor and Snider, 1934; Boyd, 1935, 1]. Up to about the middle of pregnancy, the placenta has been shown to exhibit a decreased concentration of phospholipin and an increased concentration of cholesterol esters. This indicates that the placenta declines in activity during the first half of gestation.

Lipoid analyses reveal that about the middle of gestation, the placenta begins to take on increased function, *i.e.* the percentage of cholesterol ester falls while that of phospholipin and free cholesterol rises. At the same time, fat metabolism in the foetus becomes augmented as evidenced by the laying down of great quantities of storage fat and increased concentrations of phospholipin and free cholesterol.

It would appear that these two phenomena may be related. From the data given in Figs. 1 to 4, the foetus of 16 days may be calculated to contain a total of about 6 mg. of phospholipin, 1 mg. of free cholesterol, 4 mg. of ester cholesterol and 2 mg. of neutral fat. The foetus at term may be similarly shown to contain 500 mg. of phospholipin, 75 mg. of free cholesterol, 75 mg. of ester cholesterol and 600 mg. of neutral fat. Thus it is only in the latter part of pregnancy that appreciable amounts of lipoids are segregated in the foetus. Previous studies [Boyd and Wilson, 1935] suggested that in the haemo-chorial type of placentation, the major portion of foetal lipoids is passed through the placenta. Present studies indicate that the bulk of foetal lipoids, excluding a possible portion which may be synthesised from carbohydrate, must pass through the placenta in the latter part of pregnancy. If this passage were simply one of physical transfer across the placental membranes, it is difficult to see why there should be any change in the lipoid metabolism of the placenta, especially a change which indicates increased physiological activity. Rather do the results suggest that the actual tissue substance of the placenta plays an important rôle in the transfer of lipoids from the mother to the growing offspring.

While in this series of investigations attention has been directed chiefly to the placenta as the medium through which lipoids are transferred from the mother to the embryo, it is possible that other mechanisms may exist. In animals possessing an epithelio-chorial or syndesmo-chorial type of placenta, with several layers of cells separating the maternal and foetal blood streams, *e.g.* pigs, horses and ruminants, it is difficult for the foetus to obtain sufficient nourishment directly from the maternal blood *via* the placenta and return to it waste products of metabolism. In such animals, accessory mechanisms such as "uterine milk" are provided for the nourishment of the foetus and accessory organs like the allantois for the disposal of waste products. The decidua and amniotic fluid constitute another possible mechanism for foetal nourishment.

Little is known about the relations of these several possible mechanisms to foetal fat metabolism except in the case of amniotic fluid. This fluid was found to contain but traces of fatty acids in human beings [Konstantinovitch, 1906] but appreciable amounts in the cow [Eckles, 1916]. Traces of cholesterol have been reported in human amniotic fluid [Chauffard *et al.*, 1911]. A sample of pure amniotic fluid, obtained by the author from a human ovum about two months old, gave the following lipoid analysis: neutral fat, 0; free cholesterol, 4 mg. per 100 g.; ester cholesterol, 27 mg. per 100 g.; phospholipin, 67 mg. per 100 g. It is of interest that the lipoids concerned with fat transport, *i.e.* phospholipin and cholesterol ester, were present in greatest amounts. A possible manner by which lipoids may be absorbed from amniotic fluid by the foetus can be deduced from the work of Keiffer [1926]. He found that "vernix caseosa," which is a cheesy deposit from amniotic fluid on the skin of the mammalian embryo and contains some 10 % fat, will be absorbed, if left on the skin, within eight hours of birth. These various facts suggest that lipoids may be added to amniotic fluid through the decidua and amnion, deposited on the skin of the embryo and from there absorbed by the foetus. Further work is required to substantiate this postulate.

SUMMARY.

The lipoid composition of the foetus and of the placenta was determined in the rabbit at various stages during intra-uterine development with a view to demonstrating any relation between the fat metabolism of placental tissue and fat metabolism of the foetus.

It was found that the lipoid composition both of the foetus and of the placenta varied according to the duration of growth of the foetus. In the foetus, the concentrations of phospholipin and free cholesterol increased rapidly up to the middle of gestation, then more slowly until the last week or ten days when again the rate increased. During the last ten days there was a marked increase of 600 % in the neutral or storage fat content of the foetus. The embryo contained variable but similar amounts of cholesterol ester at all stages of growth within the uterus.

Up to the mid-point of pregnancy, the placenta exhibited a fall in the concentration of phospholipin and an increased percentage of cholesterol esters.

In the latter half of gestation, the reverse changes occurred, *i.e.* the value of phospholipin rose while the value of cholesterol esters fell. Throughout pregnancy there was a slight increase in placental free cholesterol and a slight increase in placental neutral fat.

On the basis of current theory regarding the relation between lipoid composition and physiological activity, these changes were interpreted as follows. Growth maintains a high degree of physiological activity in the foetus until about the middle of pregnancy. In the last few days of gestation, the foetus again becomes active owing to an increased turnover of fat metabolism resulting in the laying down of large amounts of storage fat in the embryo. The deposition of this fat occurs along with an increased concentration of phospholipin and free cholesterol and is associated with increased activity on the part of the placenta. Such placental activity appears inconsistent with the conception that lipoids are passed through the placenta merely as through an animal membrane and suggests that the actual tissue substance of the placenta is actively engaged in transferring at least a part of the lipoids from the mother to the embryo in the latter part of gestation.

Other possible pathways for the exchange of lipoids between the mother and the foetus were discussed, particularly the decidua and amniotic fluid.

The author takes pleasure in acknowledging that he obtained the idea that the placenta may be more than a permeable membrane in relation to the acquisition of lipoids by the foetus from reading the stimulating description of this subject by Dr Joseph Needham in his book, *Chemical embryology.*

This investigation was begun while the author was associated with the Department of Obstetrics and Gynaecology, The University of Rochester, School of Medicine and Dentistry, Rochester, New York. The author desires to thank Dr A. W. Makepeace of that department for providing much of the experimental material.

REFERENCES.

Baumann and Holly (1926). *Amer. J. Physiol.* **75**, 624, 629.
Bloor (1924). *J. Biol. Chem.* **59**, 543.
—— and Snider (1934). *J. Biol. Chem.* **107**, 459.
Boyd (1933, 1). *J. Biol. Chem.* **101**, 323.
—— (1933, 2). *J. Biol. Chem.* **101**, 623.
—— (1934, 1). *J. Clin. Invest.* **13**, 347.
—— (1934, 2). *Surg. Gynaec. Obstetr.* **59**, 744.
—— (1935, 1). *J. Biol. Chem.* **108**, 607.
—— (1935, 2). *Amer. J. Obstetr. Gynaec.* (in press).
—— and Wilson (1935). *J. Clin. Invest.* **14**, 7.
Chauffard, Laroche and Grigaut (1911). *Compt. Rend. Soc. Biol.* **70**, 536, 568.
Eckles (1916). *Univ. Missouri Agric. Exp. Sta. Res. Bull.* No. 26.
Keiffer (1926). *Gynéc. et Obstétr.* **14**, 1.
Konstantinovitch (1906). Vrach. Gaz.; ref. Needham (1931). Chemical embryology, **3**, 1543. (Cambridge.)
Ling (1931). *Chinese J. Physiol.* ref. Bloor (1933), *Ann. Rev. Biochem.* **2**, 147.
Schaible (1932). *J. Biol. Chem.* **95**, 79.
Watanabe (1923). *J. Biochem.* **2**, 369.

CXX. STUDIES ON VACUUM EVAPORATION AND DISTILLATION.

By HANS NORBERT NAUMANN.

From the Devonport Pathological Laboratories, Seamen's Hospital, Greenwich.

(*Received February 5th, 1935.*)

In many chemical processes, especially in connection with modern biochemical problems, the rapid evaporation of large amounts of dilute solutions which contain heat-labile substances is necessary. For this purpose vacuum apparatus such as those of Anschütz, Hausmann and others are not efficient enough.

In recent publications the author [1929; 1930, 1, 2; 1931, 1, 2] has described efficient vacuum apparatus for rapid evaporation at low temperature. These vacuum distillation plants[1], working with water-jet pumps and giving yields of 2–10 litres of aqueous distillate per hour at temperatures of 15–20° (see Figs. 1 and 2) have the following principles in common:

(1) A highly efficient cooling system. The good condensation so produced makes for low steam pressure and boiling-point in the evaporating vessel (Fig. 1 *a*, Fig. 2 *a*).

(2) A large diameter of the tube system connecting the evaporation flask and the pump. This decreases the friction, speed and pressure of the steam flow to the lowest possible amount (Fig. 1 *b*, Fig. 2 *b*).

(3) An automatic arrangement so that the solution to be evaporated or distilled is drawn up continuously (Fig. 1 *c*, Fig. 2 *c*) and the distillate is automatically removed (Fig. 1 *d*, Fig. 2 *d*).

(4) A resistant capillary of rustless steel which can be regulated and cleaned by means of a stilette wire (Fig. 1 *e*, Fig. 2 *e*). This prevents any delay in boiling.

The apparatus now described has the further improvement, that in addition to both functions mentioned, the pump water is used also for cooling the condenser. For this purpose there is a direct connection of the outflow pipe with the condenser (Fig. 1 *f*, Fig. 2 *f*). This gives good results and, owing to the syphon action of the condenser, there is no reduction in the efficiency of the pump, if a sufficient water pressure is available. The mechanical work of the pump is not increased, and therefore the additional load of the pump is confined to the greater frictional resistance of the walls in the condenser system. When the pump is stopped the water of the condenser jacket runs into the pump and out into the vessel which may have been connected to receive the distillate. Before stopping the pump therefore either this vessel is removed or a check valve (Fig. 2 *g*) can be fitted to prevent the water reflux. The special water-jet pump working continuously and automatically has therefore three functions: (1) it evacuates the apparatus; (2) it drains off the condensate; (3) it cools the system[2].

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[1] All types of apparatus can be obtained from Messrs H. Cornbleet, 34, East Street, London, S.E. 17.

[2] Fischer [1931] considers the omission of oil pumps a disadvantage in my apparatus; but this is not in accord with my own observations as explained above. He admits, however, that the removal of the steam by oil pumps is difficult and uneconomical, and that in most cases a condenser cannot be dispensed with. Oil pumps in my opinion therefore should be confined to the domain of high vacuum distillation.

Efforts have been made in the past few years to utilise these advantages of the water-jet pump for industrial purposes as for example by Peterson and the Nederlandsche Installatie Maatschappij Therma [1925] in a British Patent for vacuum evaporation of hot inorganic salt solutions of, *e.g.*, $MgSO_4$, Na_2SO_4 *etc.* There is a similarity in the construction principle, with the exception that the condenser is erected before the pump. This arrangement however seems not to be so good because the pre-heated pump water has a higher vapour pressure which prevents an optimum vacuum.

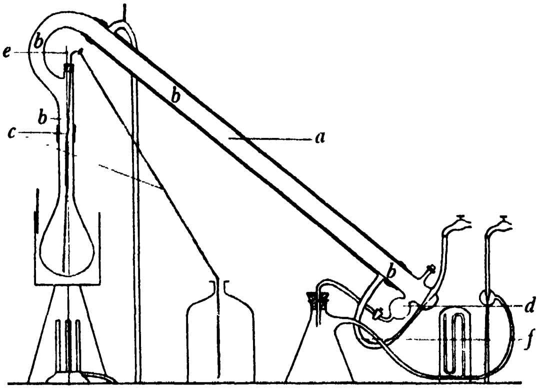

Fig. 1. Standard model of a vacuum evaporator and still for an efficiency of about 2 litres per hour at 20°.

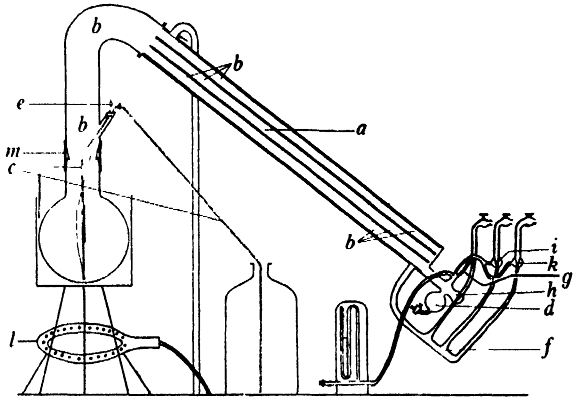

Fig. 2. Vacuum evaporator with seven-tube condenser and a continuous automatic efficiency of 5–8 litres pér hour at 20°.

The triple function of the water-jet pump is greatly extended by using a larger apparatus with seven-tube condenser (Fig. 2).

This apparatus consists of a special pump (Fig. 2 *h*) and two ordinary pumps (*i*, *k*) as auxiliary pumps, the outflow pipes of which are joined to a single tube connected to the condenser (*f*). When the evaporation is begun all three pumps are working together for the purpose of more rapid evacuation. When a suitable vacuum has been attained, the auxiliary pumps (*i*, *k*) are stopped and the evacuated system is blocked by a check valve (*g*), so that water reflux is prevented. The apparatus is then worked with this single special pump, which, notwithstanding its small size, is sufficient for fulfilling the three functions: maintaining the vacuum, draining off the condensate and cooling. The apparatus has an efficiency of 5 litres water per hour at 20°.

The economical working of the larger model is important in technical work, when it is desired to increase the evaporation efficiency to more than 5 litres per hour.

The condensation of 5 kg. of steam per hour with the use of only 300 litres of cooling water supplied by one water-jet pump is possible on account of the great surplus of the cooling surface; actually the maximum efficiency of the condenser is considerably higher. Experiments with a single copper tube condenser (Fig. 1 *a*) have shown that the condensation of 4–5 kg. of steam per hour is possible with 300 litres of cooling water. Since the larger condenser (Fig. 2 *a*) consists of seven copper tubes of the same diameter and length with a sectional cooling water surface approximately 19 times greater, an efficiency of about 30 litres per hour can be expected by using 2000 litres of cooling water. Under ordinary laboratory conditions this can hardly be effected owing to lack of sufficiently powerful heating, approximately 18,000 Cals. per hour being necessary for evaporating 30 litres water at 20°. By heat supplied directly by means of a large ring burner with 24 flames (Fig. 2 *l*) to a tinned iron boiler of 30 litres capacity, efficiencies of about 10 litres per hour have been attained.

Smaller improvements can be attained by tinning the inner surface of the condenser[1] or by using chromium-nickel-steel resistant to acid vapours and also by a conical intermediate piece connecting the evaporation vessel and the metal connecting tube (Fig. 2 *m*) as suggested independently by Hansen [1933]. As regards the boiling capillary made of rustless steel, it is to be noted that the alloy (V2*a* Krupp-Stahl) was corroded not only by hydrochloric acid, but also by more concentrated cold sulphuric acid. It has also been ascertained that a capillary tube can often be dispensed with when working with continuous inflow, since the inflowing solution contains sufficient absorbed air to prevent delay in boiling. After all the solution is in the vessel, the inflow tube can itself serve as a boiling capillary (Fig. 1 *c*, Fig. 2 *c*). But the correct regulation of the apparatus, especially at the moment when all the solution has run into the flask, demands considerable practice in order to prevent the sputtering of the solution consequent on the violent entrance of air. A boiling capillary is therefore preferable even on a continuously working apparatus.

SUMMARY.

The special water-jet pump for continuous automatic working previously described by the author has been improved, so that three functions, evacuating, cooling and removal of the condensate, are fulfilled by this one special pump.

A large apparatus has also been devised fitted with a seven-tube condenser and three pumps, in which, when a full vacuum has been obtained, a single special pump suffices for maintenance of the vacuum, cooling and draining off the condensate at an efficiency of 5 litres per hour at 20°.

The efficiency of this larger apparatus can be increased to 10 litres water per hour at 20° by using a vessel of 30 litres capacity which is heated directly. Efficiencies up to 30 litres per hour may be attained if sufficient increase of heat in proportion to the condenser capacity can be applied to the boiling vessel.

I wish to record my best thanks to Dr Arthur Davies, Director of the Devonport Pathological Laboratories of the Seamen's Hospital, Greenwich, for his help and interest in my work, to the Seamen's Hospital Society for their assistance and hospitality to me and my assistant, Miss Larissa Frenkel, whose cooperation has been of value, and to the Academic Assistance Council for a personal grant.

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[1] In one case the apparatus was silvered, which was scarcely more expensive but possibly more resistant than the tinning process.

REFERENCES.

Fischer (1931). *Chem. Fabrik*, **4**, 238.

Hansen (1933). *Acta Path. Microbiol. Scand.* Suppl. **16**, 419.

Naumann (1929). *Biochem. Z.* **216**, 136, 154.

—— (1930, 1). *Biochem. Z.* **217**, 409.

—— (1930, 2). *Chem. Fabrik*, **3**, 505.

—— (1931, 1). *Chem. Fabrik*, **4**, 4.

—— (1931, 2). *Apotheker Ztg.* 68.

Peterson and Nederlandsche Installatie Maatschappij Therma (1925). British Patent Specification, No. 243,982.

CXXI. THE PERFUSION OF THE ADRENAL GLAND WITH REFERENCE TO THE MECHANISM OF ADRENALINE STABILISATION.

By ROBERT DONALD HOSKIN HEARD
and ARNOLD DeMERRITT WELCH.

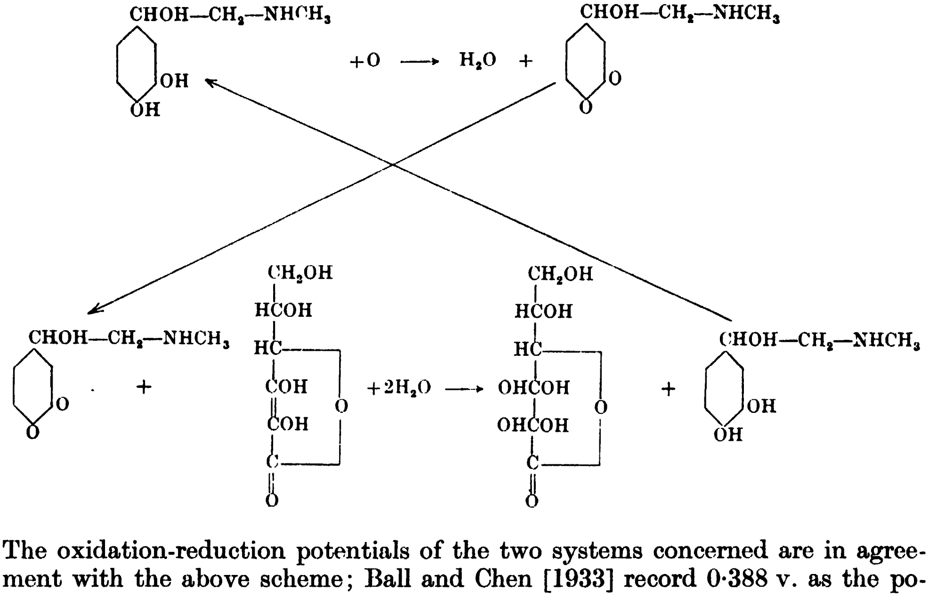
*From the Departments of Biochemistry and Pharmacology,
the University of Toronto.*

(*Received February 7th, 1935.*)

An investigation of the physiological properties of the Ringer-Locke perfusate of the adrenal gland by Schkawera and Kusnetzow [1923] revealed the presence of a sympathomimetic substance which is remarkably resistant to oxidation but in all other respects behaves as adrenaline; they concluded that a stable "adrenaline-like" substance, possibly a precursor of the hormone, is secreted on perfusion. More recently evidence was adduced [Heard and Raper, 1933] which suggested that this stable "adrenaline-like" substance of Schkawera and Kusnetzow is in reality a stabilised adrenaline. It was found that perfusion of 3:4-dihydroxyphenylalanine and its *N*-methyl derivative renders these substrates resistant to oxidation by tyrosinase, and that adrenaline, when added to the Ringer-Locke perfusate, retains its pressor activity on remaining in the alkaline medium for several days. Conceivably stabilisation of adrenaline may be effected directly by chemical combination, or by the presence of amino-acids which delay its rate of oxidation [Wiltshire, 1931], or by means of a continuous interplay of chemical reactions constituting an oxidation-reduction system. The principal purpose of the present communication is to describe results showing that the latter supposition is correct, and that, in all probability, ascorbic acid is the agent responsible for the stabilisation.

On oxidation of the perfusate by molecular oxygen at physiological hydrogen ion concentration and temperature, it was shown that pressor activity does not diminish at a steady rate as with solutions of pure adrenaline or those in which oxidation is delayed by the presence of amino-acids [Welch, 1934], but is maintained at its original level for a considerable period of time before physiological activity decreases. During this maintenance period when pressor activity remains constant, the reducing power of the perfusate, as determined by titration with 2:6-dichlorophenolindophenol, diminishes gradually and reaches zero when irreversible oxidation of adrenaline manifests itself by diminution in physiological activity and appearance of the well-known red coloration. The above observations, together with results of respirometric experiments in which it was found that the oxygen uptake curve given by the perfusate is similar to that shown by adrenaline in the presence of a reducing substance such as ascorbic acid or glutathione, indicate that stabilisation is effected by means of an oxidation-reduction system. That ascorbic acid is the agent responsible for the fixation of adrenaline may be inferred from the behaviour of the perfusate with 2:6-dichlorophenolindophenol. Harris and Ray [1933] have found that ascorbic acid is the only known naturally occurring reducing substance, with the possible exception of cysteine, which reduces this dye under the conditions employed by them. They have applied the method to the estimation of the ascorbic acid content of the adrenal gland with results in excellent agreement with those obtained by biological vitamin C assay. Consequently, with this organ, expression of the indophenol titration value in terms of ascorbic acid is seemingly justifiable; however, it should be emphasised that this does not necessarily apply to other tissue [*cf.* Young and Mitolo, 1934].

It may be assumed from the investigations of Raper and collaborators [Raper, 1932] and from potentiometric measurements recorded by Ball and Chen [1933] that the first stage in the oxidation of adrenaline involves dehydrogenation to the corresponding 3:4-quinone. The latter then rapidly undergoes further irreversible oxidation; conceivably, the quinone, at least in part, undergoes intramolecular change with the formation of an indole derivative. However, in the presence of a reducing substance of sufficiently low potential, the 3:4-quinone is reduced to adrenaline before ring formation takes place. Such a reduction of quinone was demonstrated by Szent-Györgyi [1928] with hexuronic acid and by Welch [1934] with cysteine and glutathione. The recent elucidation of the chemical structure of ascorbic acid and its primary oxidation product by Herbert *et al.* [1933] makes it possible to represent the reactions involved in the coupled system ascorbic acid-adrenaline as follows:



The oxidation-reduction potentials of the two systems concerned are in agreement with the above scheme; Ball and Chen [1933] record 0·388 v. as the potential of the adrenaline system at p_H 7 and 30° which is well above that of ascorbic acid ($E'_0 = -0{\cdot}045$ v. at p_H 7 and 30° [Green, 1933]). The coupled system has been investigated respirometrically and oxygen uptake curves obtained which are similar to those shown by the perfusates of the adrenal gland, where stabilisation of adrenaline is effected by ascorbic acid as long as a small amount of the latter persists in the reduced form. Thereafter the intramolecular change which the 3:4-quinone undergoes is somewhat delayed by the presence of amino-acids. The mechanism by which this is accomplished has been previously investigated [Welch, 1934], when it was demonstrated that the 3:4-quinone, in part, oxidatively deaminates amino-acids and is itself reduced thereby.

The fundamental rôle which ascorbic acid plays in normal metabolism has not yet been elucidated. Certainly the relatively large amounts present in both the cortex and medulla of the adrenal gland cannot be regarded as a reserve supply of vitamin C. This quantity is insufficient to supply the vitamin requirements of the body for more than a few hours. Furthermore Harris and Ray [1933] have shown that the ascorbic acid content of the gland diminishes only gradually during the whole course of scurvy, and that, at death from avitaminosis, it is not unusual to find small amounts still present in that organ. This is in marked contrast to the behaviour of the reserve supply of vitamin A held in the liver which is entirely depleted before ill-effects are observed. Inasmuch as the physiological properties of ascorbic acid appear to depend essentially upon its high reducing power and the reversible nature of its oxidation, it has been supposed that the presence of the vitamin in the adrenal gland is involved in a system which maintains adrenaline or adrenaline-like substances in a reduced state. The behaviour of the Ringer-Locke perfusate of the gland may be adduced as experimental evidence in support of this assumption. Of the reducing substances known to occur within the gland apparently ascorbic acid only is secreted on perfusion. The latter is always accompanied by adrenaline and with no perfusate was one encountered without the other. This suggests that ascorbic acid, although it represents but one component of a complex oxidation-reduction system functioning within the gland, is that reducing substance directly concerned with the maintenance of the 3:4-quinone of adrenaline in the reduced state and the inhibition of pigment formation. Its oxidation-reduction potential, which is more positive than that of glutathione and other reducing substances present in the adrenal gland, is in agreement with the above hypothesis. The effect of the vitamin on pathological pigmentation is also of interest in this connection. Szent-Györgyi [1932] states that its administration has no effect on normal pigmentation but causes the disappearance of the pathological pigmentation which accompanies adrenal insufficiency (Addison's disease). There is not secreted on perfusion a reducing substance more negative than ascorbic acid which would effect reduction of the primary oxidation product of the latter. However, there is present in the perfusate an agent which minimises the rate of autoxidation of ascorbic acid and thus tends to maintain the vitamin in the reduced form. Protection of ascorbic acid in this manner is brought about by many tissues; this has also been observed recently by other investigators [Quastel and Wheatley, 1934; De Caro and Giani, 1934; Mawson, 1934].

EXPERIMENTAL.

Perfusion of the adrenal gland.

A technique slightly modified from that previously described [Heard and Raper, 1933] and that employed by Schkawera and Kusnetzow [1923] was adopted in perfusion of this organ. The glands of an ox, together with a large piece of surrounding fatty tissue which encased 6 or 8 inches of both aorta and vena cava, were obtained from the abattoir immediately after slaughtering of the animal and conveyed to the laboratory in a Dewar flask. The inferior vena cava was opened longitudinally revealing location of the two glands, the left applied to the renal vein at its junction with the vena cava and the right directly applied to the latter immediately superior to the right renal vein. In some cases but one large vein drained each gland, whilst in others as many as five smaller vessels were found. The aorta was then sectioned in the same manner and the arterial vascularisation examined. Usually the three large lumens of the coeliac and superior mesenteric arteries and one renal artery were exposed, although occasionally there was found a common coeliac and superior mesenteric vessel which subdivided immediately on leaving the aorta. The vascularisation of the adrenal of the ox is described by Schkawera and Kusnetzow who state that two main vessels irrigate each gland; the posterior vessel arises from the aorta or renal artery whilst the anterior may branch directly from the aorta in the vicinity of the diaphragmatic artery or from any of the main aortic vessels in that region. In our experience vascularisation of the two glands is less markedly differentiated; usually two main vessels predominantly irrigating one gland could be located in the region described by Schkawera and Kusnetzow, but invariably a certain amount of cross-circulation to the second gland was encountered. In most cases adequate perfusion was obtained through an adrenal artery leading directly from the aorta; when this could not be effected, a cannula was placed in either the coeliac, superior mesenteric or renal artery, the peripheral end of which was ligatured. In order to collect perfused fluid, a section of vena cava was disengaged from surrounding tissue and tied about a large glass tube of sufficient diameter to enclose the lumens of all adrenal veins. The preparation was then placed on a heated pad maintained at 38° and oxygenated Ringer-Locke solution, warmed to this temperature, perfused at a pressure of 70–120 mm. of mercury by means of a Dale-Schuster pump. The vessel receiving the perfusate was contained in an ice-salt mixture and air was displaced by a steady stream of nitrogen.

Adrenaline and ascorbic acid contents of the perfusate.

In all experiments estimation of pressor activity was made by intravenous injection into a pithed cat; when acetylcholine was present in the fluid, the animal was previously desensitised with atropine.

Titration with 2:6-dichlorophenolindophenol using a technique slightly modified from that described by Birch *et al.* [1933] was adopted as a measure of reducing power. In order to accommodate the small amount of ascorbic acid present in the perfusate, the concentration of the dye employed was reduced to approximately 0·001 M, a procedure which increases the probable error of the determination to 5–10 %. The dye was standardised immediately before use against a commercial sample of vitamin C (B.D.H.), the reduced ascorbic acid content of which had previously been determined by titration with iodine. Indophenol titrations were carried out at p_H 2·5, which was attained either by adjustment with glacial acetic acid or by addition of an equal volume of phthalate-hydrochloric acid buffer ($M/5$) at this reaction.

The adrenaline contents of the perfusates from 15 preparations were found to vary from 10 to 0·5 mg./100 ml., which is in agreement with the values obtained by Schkawera and Kusnetzow (5–1 mg./100 ml.). This deviation may be attributed to the fact that the extent of perfusion is by no means the same in every case; on injecting methylene blue at completion of an experiment and sectioning the gland, it was seen that in some cases irrigation of as much as 70 % of the tissue was effected, in others only 20 %.

In three perfusions the effects of acetylcholine and eserine on adrenaline secretion were investigated. When added to the perfusing fluid in concentrations up to 1 in 10,000, the former gave rise to no enhanced pressor activity; however, eserine (1 in 500,000) resulted in a slight increase (40 %), and both eserine (1 in 500,000) and acetylcholine (1 in 1,000,000) significantly increased adrenaline output by 150 %. These observations are in agreement with those obtained by Feldberg *et al.* [1934] and support the hypothesis that acetylcholine is the humoral transmitter of splanchnic impulses to the adrenal medulla, so that the preganglionic sympathetic fibres are cholinergic.

In order to gain insight into the mechanism by which adrenaline present in the perfusate is stabilised and rendered resistant to oxidation, an Erlenmeyer flask of 200 ml. capacity containing perfused Ringer-Locke solution (50 ml.), was attached to the shaking arm of a water-bath maintained at 38° and constantly agitated to facilitate oxygenation. Samples were assayed and titrated at regular intervals until pressor activity had disappeared. Data from experiments performed in this manner with perfusates from several different preparations are embodied in Table I. In no case did diminution in pressor activity commence immediately; there was always found a period of some 3 to 21 hours during which time reducing power gradually diminished, whilst physiological activity remained constant (see Fig. 1). Also no development of red coloration took place until after this initial maintenance period had elapsed. This is in marked contrast to the behaviour of solutions of pure adrenaline treated in the same manner, when development of a red coloration and diminution in pressor activity commenced immediately. Adrenaline, when added to the perfusate in

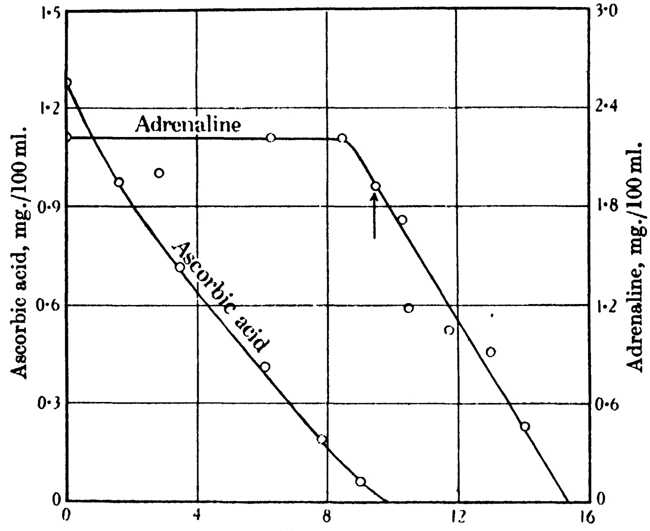


Fig. 1. Perfusate 9. Temperature 38°. Arrow indicates time when red coloration becomes apparent.

Table I.

Perfusion No.	Adrenaline content of perfusate mg./100 ml.	Maintenance period when pressor activity remains constant hours	Ascorbic acid content of perfusate mg./100 ml.	Time when indophenol titre reaches zero hours	Adrenaline content after addition of adrenaline to perfusate mg./100 ml.	Maintenance period when pressor activity remains constant hours
2	1·28	4·5	—	—	3·10	0
3	1·00	8·4	—	—	3·00	3·5
6	1·30	10·2	0·906	10·2	4·55	4·5
8	0·67	5·5	0·242	6·0	—	—
9	2·23	9·0	1·277	9·5	—	—
12	1·67	21·0	0·832	21·3	—	—
13	0·64	3·0	0·134	3·5	—	—

concentration sufficient to double or treble that originally present (see Table I) was stabilised in the same manner, although in these cases (perfusions 2, 3 and 6) the duration of the maintenance period was considerably lessened.

The above results indicate clearly that stabilisation is effected by means of an oxidation-reduction system rather than by chemical combination or amino-acids. Addition of adrenaline to the perfusate increases the concentration ratio of adrenaline to ascorbic acid so that the period during which the 3:4-quinone of the former is maintained in the reduced form (adrenaline) is considerably diminished. When the ascorbic acid is completely oxidised, irreversible oxidation of adrenaline, manifested by the appearance of a red coloration and diminution in pressor activity, commences. The rate of oxidation is now dependent upon the amino-acid content of the perfusate, and was found to vary from 0·05 to 1·06 mg. per hour. In the presence of large quantities of amino-acids, the course of adrenaline oxidation is somewhat altered; under these conditions the red coloration is replaced by a yellowish tinge.

Values ranging from 0·134 to 1·277 mg./100 ml. ascorbic acid were obtained with the various perfusates. Apparently no correlation exists between the relative amounts of ascorbic acid and adrenaline secreted on perfusion. The maximum observed ratio, in terms of molar equivalents of adrenaline to ascorbic acid, was 4·9, the minimum, 1·41; however, with 9 perfusates out of 16 examined, a value between 2 and 3 was found. In no case was the sulphydryl group detectable by the nitroprusside test either before or after reduction of the perfusate with tin and hydrochloric acid.

Respirometer experiments.

The Barcroft differential respirometer was employed in these experiments.

The stabilisation of adrenaline by an oxidation-reduction system. The coupled systems adrenaline-ascorbic acid and adrenaline-glutathione were investigated. At physiological hydrogen ion concentration and temperature, both adrenaline and ascorbic acid rapidly undergo autoxidation; in order to obtain total oxygen consumption, phosphate buffer solution at p_H 7·36 (2·25 ml. of $M/5$) was placed in the side-arm of the Barcroft vessel and, after equilibration in the water-bath at 38°, spilled into solutions of *l*-adrenaline hydrogen tartrate and ascorbic acid contained in the main body of the flask. The required amounts of solutions of ascorbic acid containing 0·88 mg. (1/200 millimol.) per 0·25 ml. and of adrenaline hydrogen tartrate containing 0·916 mg. (1/200 millimol.) of free adrenaline per 0·25 ml. were added to the main body of the reaction vessel to give the proportions indicated in Fig. 2; where necessary distilled water was added to a total volume of 3 ml. In all cases the compensator vessel contained 3 ml. of buffer solution only. In order to absorb carbon dioxide, a small pledget of cotton-wool was placed within the ground glass joint of the respirometer and moistened with potassium hydroxide solution (0·2 ml. of 12 %). In the same manner oxidation of adrenaline in the presence of the quantities of glutathione indicated in Fig. 3, was investigated. However, in these experiments it was necessary to modify the technique slightly in order to accommodate sufficient glutathione to give an optimum uptake of oxygen and yet preserve a favourable proportion of adrenaline. Only 1 ml. of $M/5$ buffer was used and maintenance of reaction at p_H 7·36 was ensured by addition to this buffer (contained in the side-arm of the flask) of potassium hydroxide solution (0·31 N) in quantity sufficient to neutralise the amount of adrenaline hydrogen tartrate employed. The amount of alkali required was determined previously by titration to p_H 7·4 (bromo-thymol blue). On spilling the contents of the side-arm into the flask after equilibration, the side-arm was washed out several times with the contents of the flask in order to ensure adequate mixing.

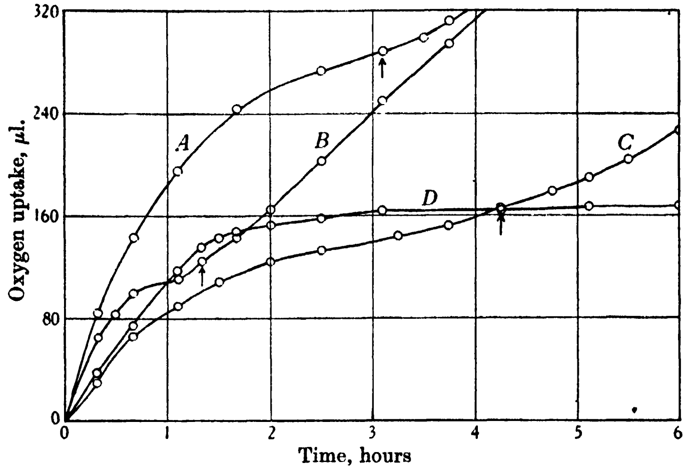


Fig. 2. Ascorbic acid-adrenaline at 38° and p_H 7·36.

Curve *A*. Ascorbic acid (1/100 millimol.)-adrenaline (1/200 millimol.).
Curve *B*. Ascorbic acid (1/200 millimol.)-adrenaline (1/100 millimol.).
Curve *C*. Ascorbic acid (1/200 millimol.)-adrenaline (1/200 millimol.).
Curve *D*. Ascorbic acid only (1/200 millimol.).

One atom of oxygen per molecule of ascorbic acid = 56 μl. per 1/200 millimol. Arrow indicates time when red coloration becomes apparent.

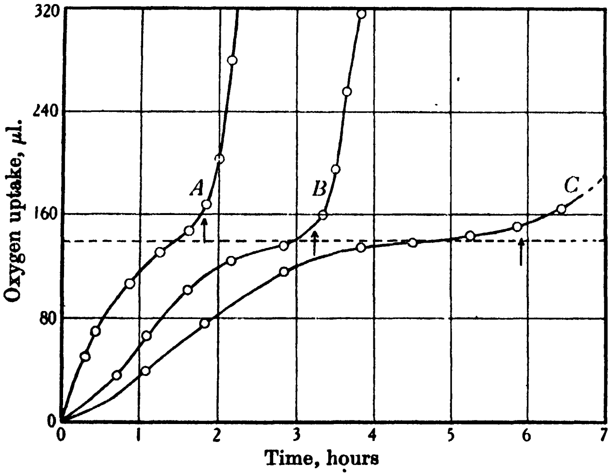


Fig. 3. Glutathione-adrenaline at 38° and p_H 7·36.

Curve *A*. Glutathione (1/40 millimol.)-adrenaline (1/8 millimol.).
Curve *B*. Glutathione (1/40 millimol.)-adrenaline (1/40 millimol.).
Curve *C*. Glutathione (1/40 millimol.)-adrenaline (1/166 millimol.).

One-half atom of oxygen per molecule of glutathione = 140 μl. Arrow indicates time when red coloration becomes apparent.

The oxygen uptake curves obtained are those characteristic of a reversible oxidation-reduction system. With glutathione (Fig. 3) differentiation of the component curves expressing rate of oxidation of reductant and oxidant respectively is clearly defined; approximately one-half atom of oxygen per molecule of glutathione (theoretical 140 μl.) is absorbed before the lag phase is encountered and irreversible adrenaline oxidation commences with the appearance of a red coloration. However, with ascorbic acid (Fig. 2) demarcation is less evident, which may be attributed to the fact that the reversible primary oxidation product of ascorbic acid is capable of undergoing further oxidation rapidly [Herbert *et al.*, 1933], whereas oxidised glutathione is relatively stable under experimental conditions. The total oxygen uptake of ascorbic acid alone (curve *D*, Fig. 2) is approximately 3 atoms of oxygen per molecule of ascorbic acid (theoretical 168 μl.), whilst irreversible oxidation of adrenaline takes place before this quantity of oxygen is utilised. A control experiment in which a solution of ascorbic acid (0·033 %) in phosphate buffer at p_H 7·4 (15 ml. of $M/5$) was shaken in the same bath with the Barcroft apparatus showed that the reducing power, as determined by titration with 2:6-dichlorophenolindophenol, reached zero when approximately 2·4 atoms of oxygen per mol. of ascorbic acid had been absorbed, which is in fair agreement with the time at which irreversible oxidation of adrenaline begins.

Behaviour of the perfusate. When examined respirometrically, all perfusates gave oxygen uptake curves characteristic of an oxidation-reduction system. However, in this instance ample evidence was obtained which suggests that the system is complicated by uncontrollable factors and is not strictly comparable with a reaction mixture containing ascorbic acid and adrenaline only. Of 9 perfusates investigated in this manner, 6 showed immediate utilisation of oxygen whilst a lapse of several hours was encountered with the remainder before an appreciable quantity of oxygen was consumed. The curve shown in

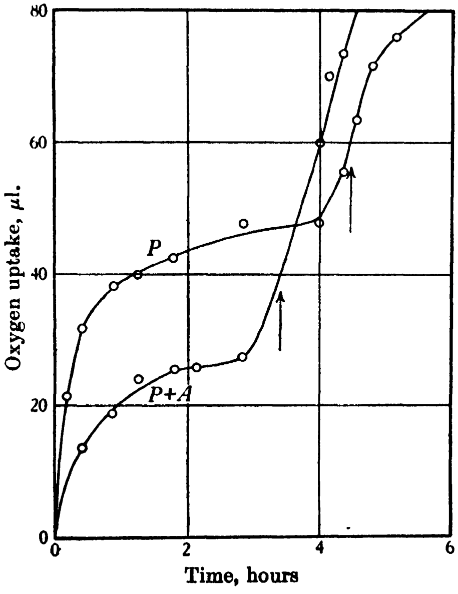


Fig. 4.

Curve *P*. Perfusate 6 (10·0 ml.): 1·30 mg./100 ml. adrenaline-0·91 mg./100 ml. ascorbic acid.
Curve *P*+*A*. Perfusate 6 (9·86 ml.) + adrenaline (0·143 ml. of 1 in 1000): 2·70 mg./100 ml. adrenaline-0·9 mg./100 ml. ascorbic acid.

Temperature 38°. Arrow indicates time when red coloration becomes apparent.

Fig. 4 illustrates the behaviour of a perfusate from the former group. The example cited is typical, and any deviation from this type of curve was manifested by lesser demarcation of the components expressing rates of oxidation of ascorbic acid and adrenaline, which in some cases was detectable only by development of a red coloration signifying irreversible oxidation of the latter; as demonstrated above, this may be explained by the relative proportions of ascorbic acid and adrenaline present. Again it is evident that addition of a further quantity of adrenaline to that originally contained in the perfusate (curve $P+A$) accelerates the rate of oxidation of ascorbic acid and diminishes the period during which adrenaline is maintained in the reduced form. It is also apparent that the rates of oxidation obtained respirometrically and by indophenol titration are not commensurate. For example, with perfusate 6 irreversible oxidation of adrenaline begins in the Barcroft vessel within 4·5 hours (Fig. 4), whilst approximately 10 hours elapse before diminution in physiological activity becomes noticeable (see Table I). At least two factors contribute to this irregularity; first, comparison of the rate of oxidation of a small volume of perfusate in the respirometer with that of a larger volume contained in an Erlenmeyer flask is not justifiable where conditions of surface area *etc.*, are radically altered, and second, removal of carbon dioxide from the atmosphere surrounding feebly buffered Ringer-Locke solution in the Barcroft vessel renders the reaction considerably more alkaline. Several unsuccessful attempts were made to establish conditions such that oxidation would proceed at the same rate in each case. The presence of phosphate buffer renders the end-point of the titration with the dye extremely indefinite, and in the respirometer, the difference between rates of oxidation with and without CO_2 absorption is not sufficiently great to account for the observed discrepancy.

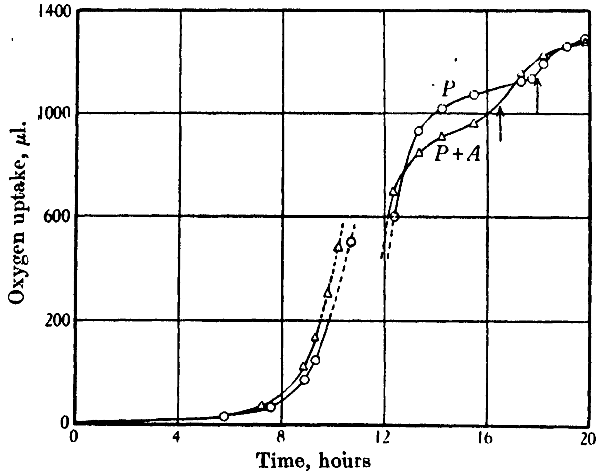


Fig. 5.

Curve P. Perfusate $2X$ (3·0 ml.): 8·06 mg./100 ml. adrenaline.
Curve $P+A$. Perfusate $2X$ (2·8 ml.) + adrenaline (0·2 ml. of 1 in 1000): 14·17 mg./100 ml. adrenaline.

Temperature 28°. Arrow indicates time when red coloration becomes apparent.

The oxygen uptake curve shown in Fig. 5 is representative of the second group of three perfusates which displayed a lag phase and illustrates the behaviour of one of the earlier perfusates obtained by cross-circulation to the second gland. In this instance perfusion for 4 hours yielded 6 ml. only; hence it was not possible to follow the rate of oxidation of ascorbic acid by indophenol titration or that of adrenaline physiologically, and thereafter a perfusate of such high potency was not encountered.

The lapse of several hours before an appreciable amount of oxygen is utilised indicates the presence of a substance or mechanism which delays oxidation of ascorbic acid. In this connection, the effects of other tissues on the inhibition of ascorbic acid oxidation have been examined respirometrically and the widespread occurrence of an inhibitor throughout the animal body noted. At p_H 7·36 and 38°, the Q_{O_2} of ascorbic acid (defined as μl. of oxygen consumed per mg. per hour) is approximately 100, whilst in the presence of a trichloroacetic acid (2·5 %) extract of liver (corresponding to 0·1 g. of wet tissue) this value is reduced to 4·5. Similarly the rate of diminution of reducing power, as determined by titration with 2:6-dichlorophenolindophenol and under the conditions defined above, is equivalent to 0·95 mg. per hour with ascorbic acid alone and 0·05 in the presence of the same quantity of liver extract.

With the various perfusates examined respirometrically, it has been found that the amount of oxygen consumed before irreversible oxidation of adrenaline begins is not proportional to the ascorbic acid content of those perfusates. This value (Figs. 4 and 5) is many times greater than the theoretical 2–3 atoms of oxygen per molecule of ascorbic acid obtained with a reaction mixture containing ascorbic acid and adrenaline only (Fig. 2). This aspect of the problem is being further investigated; preliminary experiments have shown that the primary oxidation product of ascorbic acid is capable of oxidising certain other substances such as pyruvate and amino-acids, so that the total oxygen uptake under these conditions is considerably enhanced.

SUMMARY.

1. An investigation of the chemical and physiological properties of the Ringer-Locke perfusate of the adrenal gland has confirmed secretion of a stable sympathomimetic substance.

2. The pressor activity of the perfusate may be attributed to its adrenaline content. Adrenaline, when added to perfused fluid, is likewise stabilised and rendered resistant to oxidation.

3. Stabilisation is effected by means of an oxidation-reduction system. Judging from reduction of 2:6-dichlorophenolindophenol, ascorbic acid is the agent responsible for the maintenance of the 3:4-quinone of adrenaline in the reduced state and the inhibition of pigment formation. As long as a small amount of ascorbic acid remains present in the reduced form, diminution in pressor activity does not take place; thereafter oxidation of adrenaline is somewhat delayed by the presence of amino-acids.

4. The oxygen uptake curve given by the perfusate is characteristic of an oxidation-reduction system and is similar to those obtained with the coupled systems ascorbic acid-adrenaline and glutathione-adrenaline which have been investigated respirometrically.

5. On perfusion, there is not secreted any reducing substance more negative than ascorbic acid; however, the perfusate does contain an agent which minimises the rate of autoxidation of ascorbic acid and thus tends to maintain the latter in the reduced state. Protection of ascorbic acid in this manner is effected by cell-free extracts of several tissues.

Our thanks are due to the Banting Research Foundation for defrayment of expenses in part and to Prof. V. E. Henderson for facilities provided and advice; one of us (R. D. H. H.) is indebted to the Foundation for a personal grant and to Prof. H. S. Raper for helpful suggestions.

REFERENCES.

Ball and Chen (1933). *J. Biol. Chem.* **102**, 691.
Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
De Caro and Giani (1934). *Z. physiol. Chem.* **228**, 13.
Feldberg, Minz and Tsudzimura (1934). *J. Physiol.* **81**, 286.
Green (1933). *Biochem. J.* **27**, 1044.
Harris and Ray (1933). *Biochem. J.* **27**, 2006.
Heard and Raper (1933). *Biochem. J.* **27**, 36.
Herbert, Hirst, Percival, Reynolds and Smith (1933). *J. Chem. Soc.* 1270.
Mawson (1934). *Chem. and Ind.* **53**, 1027.
Quastel and Wheatley (1934). *Biochem. J.* **28**, 1014.
Raper (1932). *Ergebn. Enzymforsch.* **1**, 270.
Schkawera and Kusnetzow (1923). *Z. ges. exp. Med.* **38**, 37.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
—— (1932). *Deutsch. med. Woch.* **58**, 852.
Welch (1934). *Amer. J. Physiol.* **108**, 360.
Wiltshire (1931). *J. Physiol.* **72**, 88.
Young and Mitolo (1934). *Nature*, **133**, 572.

CXXII. THE ACTION OF IODOETHYL ALCOHOL ON THIOL COMPOUNDS AND ON PROTEINS.

By DAVID ROCKWELL GODDARD[1]
and MAXWELL PHILIP SCHUBERT.

From the laboratories of The Rockefeller Institute for Medical Research, New York.

(*Received February 15th, 1935.*)

Mowat and Stewart [1934] have made a study of the inhibitory action of iodoethyl alcohol on the rate of disappearance of glucose from shed blood. Iodoethyl alcohol appears to be only about half as efficient as iodoacetic acid in inhibiting the glycolysis. The further observation is made that although iodoacetic acid reacts with glutathione, iodoethyl alcohol appears not to do so at all. The conclusion is drawn that although iodoacetic acid may inhibit glycolysis by interaction with thiol groups of the enzyme, the mechanism of inhibition by iodoethyl alcohol must be different.

It is the purpose of the present work to show that iodoethyl alcohol does react with thiol groups in the same way as iodoacetic acid [Dickens, 1933; Michaelis and Schubert, 1934]. In the first place the compound

$HO.CH_2.CH_2.S.CH_2.CHNH_2.COOH$, *S*-($\beta$-hydroxyethyl)cysteine,

has been isolated in crystalline form and identified by analysis and properties. It was prepared by the action at room temperature of iodoethyl alcohol on cysteine in aqueous solution at a p_H in the bicarbonate-carbonic acid range.

To show that iodoethyl alcohol also reacts with glutathione it was thought sufficient not to isolate the compound as in the case of cysteine but merely to measure the decrease with time in iodine titre of a solution containing glutathione

Table I.

Each experiment is made with 50 ml. of *M* phosphate buffer with enough added KOH solution to neutralise added cysteine hydrochloride, glutathione or iodoacetic acid. Either 0·4 g. cysteine hydrochloride or 0·7 g. glutathione is dissolved in this buffer and if an iodo-compound is to be added, 0·5 g. of either iodoacetic acid or iodoethyl alcohol. At the time intervals mentioned 10 ml. samples are withdrawn and dropped into 5 ml. of 25 % acetic acid. The samples are titrated with 0·0406 *M* alcoholic iodine, the number of ml. of this solution being recorded in the table. After the last sample has been withdrawn the p_H is measured with a glass electrode.

ml. iodine solution used.

Time mins.	Cysteine			Glutathione		
	Alone	$+CH_2I.CH_2OH$	$+CH_2I.COOH$	Alone	$+CH_2I.CH_2OH$	$+CH_2I.COOH$
0·5–1	12·9	12·9	3·3	10·7	10·7	3·1
3	—	—	0·7	—	—	0·7
6	—	—	0·2	—	—	0·3
11	—	—	0·0	—	—	0·1
75	12·9	11·0	—	10·5	9·3	—
160	12·8	9·6	—	10·2	7·9	—
280	12·0	8·0	—	9·8	5·8	—
p_H	7·70	7·64	7·55	7·70	7·65	7·57

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[1] National Research Fellow in the Biological Sciences.

and iodoethyl alcohol at p_H 7·5. This rate of decrease is of about the same order as with cysteine in which case the compound itself had been isolated. The data are given in Table I. It is at once evident that although iodoethyl alcohol reacts with the thiol group of cysteine and glutathione it does so at a rate very much slower than iodoacetic acid. Some similar experiments were made with iodoacetamide and showed that this substance reacts as quickly with either cysteine or glutathione as does iodoacetic acid, but since the figures have no value except to show roughly the speed of the reaction they are not included here.

15 g. $NaHCO_3$ are placed in an Erlenmeyer flask through which a steady stream of nitrogen is passed. Through a dropping funnel is added a solution of 6·4 g. cysteine hydrochloride in 15 ml. water, and then 7·2 ml. iodoethyl alcohol are washed in with 15 ml. more water. The mixture is allowed to stand 12 hours. The solution is then acidified with HCl and neutralised to Congo red paper with NaOH. It is now evaporated to dryness *in vacuo* and the residue extracted with 75 to 100 ml. of 85 % alcohol. The mixture is filtered and to the filtrate about 500 ml. acetone are added. A white crystalline precipitate forms. This may be recrystallised several times more by precipitation from 60 to 80 % alcohol with acetone. The product is dried *in vacuo*. Found C, 36·61; H, 6·32; N, 8·27; S, 19·13 %. $C_5H_{11}O_3NS$ requires: C, 36·36; H, 6·72; N, 8·49; S, 19·39 %.

That the iodoethyl alcohol has reacted with the SH group and not the NH_2 group of the cysteine is evident from the negative nitroprusside test.

Behaviour with proteins. Mirsky and Anson [1935] have shown that iodoacetic acid reacts completely with the thiol groups of certain proteins. The data listed in Table II show that iodoethyl alcohol and iodoacetamide react similarly with a thiol-protein, but that the iodoethyl alcohol does not react as rapidly as does iodoacetic acid or iodoacetamide.

The protein used for the experiments is a protein prepared from wool by reduction with alkaline thiolacetate [Goddard and Michaelis, 1934]. This protein contains about 12 % cystine, and may easily be reduced. 1·75 g. of dry protein and 50 ml. of molar phosphate buffer (p_H 7·4), containing 1 milliequivalent of neutralised thiolacetic acid, are added to each of 4 flasks through which a steady stream of N_2 is passed. Two hours are allowed for reduction of the proteins. (The proteins are not completely dissolved, but solid proteins are reduced by thiolacetate, and react with iodo-compounds.) To three of the flasks 50 ml. of *M* phosphate buffer (p_H 7·4) containing 2 milliequivalents of either iodoethyl alcohol, iodoacetamide or neutralised iodoacetic acid, are added. The fourth flask serves as a control. After 2 hours the p_H is determined with a glass electrode, and the proteins are then brought to the turning point of methyl red with acetic acid. The proteins are then dialysed for 3 days against running water. Samples of these proteins are prepared for analysis by precipitation with trichloroacetic acid, washed with acetone and acid acetone and dried in a vacuum desiccator [see Mirsky and Anson, 1935]. The cystine analyses are made by the method of Folin and Marenzi [1929], after oxidising the acid hydrolysate with 3 % H_2O_2. Moisture content is determined by drying to constant weight at 105°. A similar experiment was carried out at higher p_H using sodium veronal as buffer.

The reduction of the proteins by thiolacetic acid is not complete, as is shown by the analyses for residual cystine given in Table II. When the reduction is carried out at a higher p_H the reduction is more complete as is shown by the lower residual cystine values. The reaction of the iodo-compounds with the thiol groups was complete in all preparations except that with iodoethyl alcohol at p_H 7·4, this preparation alone gave a positive nitroprusside test on the protein dialysed free from thiolacetic acid.

Table II.

Figs. % based on dry weight of the proteins.
Proteins dried at 105° to constant weight.

Protein	Cystine	Sulphur	Nitrogen
A. Wool protein (control)	12·1, 12·3	3·21	15·9
B. Prepared from A by iodoacetate at			
p_H 7·4–6·99	2·06	3·31	15·7
p_H 9·5–8·6	1·42	—	—
C. Prepared from A by iodoacetamide at			
p_H 7·4–7·33	2·25	3·25	14·8
p_H 9·5–8·8	1·7	—	—
D. Prepared from A by iodoethyl alcohol at			
p_H 7·4–7·1	6·25	3·29	14·5
p_H 9·5–9·0	1·27	—	—

DISCUSSION.

It thus appears that iodoethyl alcohol reacts with the thiol groups of cysteine and glutathione or of proteins derived from wool just as does iodoacetic acid, only the reaction is much slower. With the wool proteins this slowness is less apparent, since even at p_H about 7, half of the SH of the protein has reacted in 2 hours. With cysteine or glutathione on the other hand in 2 hours less than 25 % of the SH group has reacted. Mowat and Stewart, in testing the action of iodoethyl alcohol on glutathione, allowed only 1 hour, while in testing the action on glycolysis the reaction covered 5 to 7 hours. This coupled with the fact mentioned above that iodoethyl alcohol, in the case studied, reacts faster with protein SH than with glutathione may easily account for the apparent findings of Mowat and Stewart. Therefore we do not believe that the existing evidence requires that a new assumption be made that the mechanism of inhibition by iodoethyl alcohol is different from that by iodoacetic acid.

SUMMARY.

It has been shown that iodoethyl alcohol reacts with SH groups of cysteine, glutathione and proteins derived from wool. There is no need to assume different mechanisms of inhibition of glycolysis by iodoacetic acid and by iodoethyl alcohol.

REFERENCES.

Dickens (1933). *Biochem. J.* **27**, 1141.
Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Goddard and Michaelis (1934). *J. Biol. Chem.* **106**, 605.
Michaelis and Schubert (1934). *J. Biol. Chem.* **106**, 331.
Mirsky and Anson (1935). *J. Gen. Physiol.* **18**, 307.
Mowat and Stewart (1934). *Biochem. J.* **28**, 774.

CXXIII. A NEW METHOD FOR THE DETERMINATION OF FRUCTOSE IN BLOOD.

By LESLIE DOUGLAS SCOTT.

From the Department of Biochemistry, Charing Cross Hospital, Institute of Pathology.

(*Received March 6th, 1935.*)

A considerable experience in the routine practice of the fructose tolerance test has amply demonstrated the inadequacy and unsatisfactory nature of the test in determining liver insufficiency when the response is measured by the changes in the level of the total reducing sugar of the blood, resulting from the ingestion of fructose. The defects of the test in its present form will be fully discussed in a subsequent paper from this laboratory and need not be elaborated here except to say that they led to the recognition that it was eminently desirable to attempt to determine the actual fructose in the blood following the ingestion of this sugar in place of the ordinary values for the total reducing sugar content. The methods most generally adopted for the purpose of estimating the true fructose content of the blood are those based on the Van Creveld principle using the colour developed in the reaction with dimethylamine [Radt, 1928], whilst an entirely different principle, biological in character, has recently been used by Harding *et al.* [1933].

An alternative colorimetric method emerged from a previous study of the interaction of bile salts and fructose. It was first suggested by Dr J. Patterson that an attempt should be made to adapt the bile salt reaction described by Scott [1934] in order to apply it for the determination of fructose in blood, that is, instead of an excess of fructose being utilised for the estimation of minute amounts of bile salts, the latter might, it was thought, be employed in a similar manner to react with minute quantities of fructose. On trial it was found that a similar reddish purple colour still resulted as in the original reaction, so that it was subjected to a detailed study and has been made the basis of the method for fructose determination which is outlined below.

Reagents and apparatus:

(1) 10 % zinc sulphate solution.

(2) 0·5N sodium hydroxide.

(3) 1 % (by volume) acetic acid.

(4) 1 % stock fructose (Merck or B.D.H. glucose-free) in a saturated solution of benzoic acid.

(5) Absolute alcohol.

(6) 2·25 % alcoholic sodium tauroglycocholate (B.D.H.).

(7) Concentrated hydrochloric acid (B.D.H. "Analar").

(8) Filter-papers: No. 41, 9 cm., and No. 50, 7 cm. (Whatman).

(9) Monax boiling-tubes, 25 × 150 mm.

METHOD.

Into a boiling-tube (25 × 150 mm.), graduated at 40 ml., place 5 ml. of whole blood. Then add about 30 ml. of water, followed by 5 ml. of 10 % $ZnSO_4$ and 5 ml. of 0·5N NaOH. Mix well after the addition of each. Dilute both to the 40 ml. mark and again mix thoroughly. Place in the water-bath at 80–85° for

5 minutes, noting that the water in the bath completely covers the volume of the blood mixture in the tube. At the end of 5 minutes cool very thoroughly in running water and mix well. Filter through a No. 41, 9 cm. paper, stirring gently the remaining gelatinous precipitate on the filter-paper to free the last few ml. of filtrate. Transfer 20 ml. of clear filtrate to one of the monax boiling-tubes, add 3 drops (0·15 ml.) of 1 % acetic acid and a few silica chips to prevent bumping in the subsequent evaporation and carefully boil down to about 1·5 ml. When the fluid decreases in volume avoid excessive heat so as not to char the solution. Prepare the standard by adding to a similar monax tube 2·5 ml. 0·01 % fructose solution (by dilution of the 1 % stock fructose). Carefully boil off the excess water until the volume is about 1·5 ml. To the standard and unknown add 3 ml. 2·25 % alcoholic sodium tauroglycocholate. After the addition of an extra 7–10 ml. absolute alcohol and a few more silica chips to each tube, evaporate to complete dryness on the boiling water-bath. If bumping should occur in the blood filtrate tube owing to the precipitate which forms on the addition of the alcoholic bile salts, gently rotate the tube until all the alcohol has been evaporated. After the last drops of alcohol have disappeared allow the tubes to remain in the water-bath an extra minute or two to ensure the complete removal of the last traces of alcohol, which should be accomplished without causing the precipitate to adhere too firmly to the bottom and sides of the boiling-tube. When cool add 10 ml. concentrated HCl to both tubes, and after mixing well, securely stopper the tubes. Place in the water-bath or a beaker of water at 40° for half an hour with occasional agitation to ensure an even emulsion and production of colour. A reddish purple colour, proportional to the amount of fructose present, slowly develops, reaching a maximum intensity in 30 minutes. Remove the tubes from the bath and cool very thoroughly in running water. Filter the coloured solutions through No. 50, 7 cm. papers, refiltering the first 2 or 3 ml. as these are usually slightly cloudy. Compare in the colorimeter in the usual way. Calculation:

$$\frac{\text{Standard}}{\text{Unknown}} \times \text{Strength of standard (0·25 mg.)} \times \frac{100}{2·5} = \text{mg. fructose per 100 ml. blood.}$$

Table I. *Showing the quantitative recovery of various quantities of fructose added to normal whole blood.*

Standard employed	Amount of fructose added per 100 ml. normal blood	Amount of fructose recovered mg. per 100 ml.
1 ml. (1 mg.) of 1 in 10 dilution of 1 % fructose	50	50
	25	27
2·5 ml. (0·25 mg.) of 1 in 100 dilution of 1 % fructose	15	15
	10	10
	5	6
Using as standard 20 ml. (equiv. 2·5 ml. blood) of normal blood-filtrate to which had been added the requisite amount of fructose (see above)	50	48
	25	24
	15	14
	10	10
	5	5

When for various reasons it is impossible to obtain venous samples, the test can be carried out on 2 ml. whole blood obtained by finger puncture with only slight modification of the original technique. The essential difference can be summarised as follows:

Into a test-tube, graduated at 20 ml., place 10 ml. water, 2 ml. blood and corresponding amounts of precipitating reagents. After diluting to 20 ml. with water place in the water-bath at 80° for 5 minutes. Cool and filter through a No. 41, 9 cm. paper. Measure 10 ml. filtrate into a monax tube, add 2 drops of 1 % acetic acid and boil down to about 1 ml. After the addition of 1 ml. alcoholic bile salts *plus* 7–10 ml. alcohol, evaporate to dryness on the boiling water-bath. The standard is prepared by taking 1 ml. of a 1 in 100 dilution of 1 % fructose *plus* 1 ml. bile salt solution and 7–10 ml. alcohol. Take to dryness on water-bath. 5 ml. concentrated HCl are added to each tube, after which the tubes are heated at 40° for 30 minutes. Cool and filter through No. 50, 7 cm. papers. Compare the colours in a micro-colorimeter or by small graduated test-tubes suitable for dilution colorimetry. Calculation:

$$\frac{S}{U} \times 10 = \text{mg. fructose/100 ml. blood.}$$

Quantitative figures again show a complete recovery of added fructose.

Comparison of the capillary blood with venous samples shows that the actual fructose content is a shade higher in the former than in the latter, the difference being about 2 mg. per 100 ml. (Table II). The fructose curve thus shows the same tendency, though not nearly so marked, as does the total reducing sugar curve, in rising to a slightly higher level in the capillary than in the venous blood. Different standards of normality must therefore be accepted for the different types of blood used. In the single normal case studied in detail where the fructose contents of venous and capillary blood show close correspondence, the blood sugar levels in the two display much greater divergence.

Table II. *Showing the difference between the venous and capillary fructose in a normal subject; also a case of liver disease complicated by diabetes after the ingestion of 50 g. pure fructose.*

	Venous		Capillary	
Case	Actual estimated fructose mg./100 ml.	Total reduction (MacLean's method) mg./100 ml.	Actual estimated fructose mg./100 ml.	Total reduction (MacLean's method) mg./100 ml.
Normal subject:				
Before fructose	0	102	0	105
1 hour after	8	102	9	121
2 hours after	5	97	3	92
Subacute yellow atrophy and diabetes:				
Before fructose	0	184	—	—
1 hour after	16	232	—	—
2 hours after	13	221	—	—
After 2 months when the jaundice had entirely disappeared:				
Before fructose	0	176	—	—
1 hour after	12	240	—	—
2 hours after	7	217	—	—

Note. For the sake of convenience it might be mentioned that the average normal response in terms of actual fructose is 10 mg. and 5 mg. per 100 ml. blood in the 1 and 2-hour samples after fructose respectively.

A case of liver disease complicated by diabetes has been included in Table II for the purpose of illustrating the influence of diabetes on the actual fructose curve. The disturbance from the normal due to diabetes appears to be of the order of 2 mg. per 100 ml. in the 1 and 2-hour samples of venous blood after the administration of 50 g. pure fructose.

Table III shows a comparison of the fructose content of the blood following the ingestion of 50 g. Merck's pure fructose in normal subjects with that of

Table III.

Series No.	Type of case	Venous samples		
		New fructose method mg. per 100 ml.		Old fructose method. Total reduction (MacLean) mg. per 100 ml.
1	Normal	B.L.	0	53
		½ hour	10	70
		1 hour	10	70
		1½ hours	7	85
		2 hours	4	76
		3 hours	1	69
2	—	B.L.	0	92
		½ hour	12	92
		1 hour	12	95
		1½ hours	9	90
		2 hours	5	92
3	—	B.L.	0	85
		½ hour	8	88
		1 hour	9	86
		1½ hours	8	86
		2 hours	6	87
4	Acute alcoholic intoxication of liver	B.L.	0	109
		½ hour	12	135
		1 hour	12	143
		1½ hours	11	141
		2 hours	11	139
5	N.A.B. jaundice	B.L.	0	96
		1 hour	18	110
		2 hours	15	104
6	Subacute yellow atrophy and diabetes	B.L.	0	184
		1 hour	14	232
		2 hours	11	221

patients suffering from certain forms of liver disease. The alteration in the total reducing sugar as estimated by MacLean's method is also included for purposes of comparison.

A full survey of the results obtained by this method and their relation to the carbohydrate metabolism and liver function will, it is hoped, be ready for publication shortly.

If the amounts of fructose shown here as appearing in normal blood after the ingestion of 50 g. fructose be compared with the corresponding values found by the biological method of Harding *et al.*, they are seen to be of about the same order (actual figures are not given there but have to be arrived at by inspection of the recorded chart). On the whole the biological values are a little smaller and show the disappearance of fructose from the blood at an earlier stage than does the colorimetric method. On the other hand almost exact agreement has been obtained between the present method and a modification of the original Radt diphenylamine method [Patterson, unpublished results].

SUMMARY.

1. A new method is described for the estimation of fructose in blood based on the reaction between bile salts and fructose. The delicacy of the reaction is such that fructose contents of the order of 5–20 mg. per 100 ml. can be estimated with an error of approximately ± 1 mg. Using the method comparisons have been made between venous and capillary blood and also between normal and diabetic blood following the ingestion of fructose.

2. Preliminary results also show that a more satisfactory fructose tolerance test for liver function can be made on the basis of specific fructose estimations, than by taking account of the total blood sugar values.

I wish to thank Dr J. Patterson for his encouragement and advice throughout the course of this work, and also Dr R. A. Hickling, Physician to Charing Cross Hospital for his kindness in allowing access to his wards for the liver cases.

REFERENCES.

Harding, Nicholson and Armstrong (1933). *Biochem. J.* **27**, 2035.
Radt (1928). *Biochem. Z.* **198**, 195.
Scott (1934). *J. Lab. Clin. Med.* **19**, 523.

CXXIV. THE EFFECT OF γ-RAY IRRADIATION UPON THE GROWTH AND NITROGENOUS METABOLISM OF THE PROTOZOON, *BODO CAUDATUS*.

By NORMAN RISBOROUGH LAWRIE[1]
AND MURIEL ROBERTSON.

From the Bonnett Memorial Laboratory, Addenbrooke's Hospital, Cambridge, and the Lister Institute, London.

(*Received March 30th, 1935.*)

DATA concerning the retardation of the multiplication of growing cultures of *Bodo caudatus* when exposed to γ-radiation were recorded in a previous communication by one of us [Robertson, 1932]. The biological reactions to this radiation, the nature of this inhibiting effect and a consideration of the sensitive period in the life of the cell and of its behaviour upon removal from the radium, form the subject of a detailed study which will be recorded elsewhere [Robertson, 1935]. The reader is referred to this paper for particulars of the technique used for the culture, counting and measurement of the organism.

The purpose of the present communication is to record certain effects of γ-radiation upon the nitrogenous metabolism of *Bodo caudatus* and to correlate these effects with the morphological changes which were simultaneously produced. It has been shown in a previous communication [Lawrie, 1935] that the chief end-product of nitrogenous metabolism of a culture of *Bodo caudatus* fed upon a suspension of bacteria in an inorganic medium is ammonia. The method of estimation of ammonia by deproteinisation and nesslerisation there described has been used in the present work. The rate of production of ammonia has been taken as an index of the rate of nitrogen metabolism in the cultures.

There is little previous work which bears directly upon our experiments, because previous studies of the biological and biochemical effects of irradiation have usually been concerned with the effects following the irradiation rather than with those contemporaneous with the irradiation.

Crabtree [1932] irradiated Jensen Rat Sarcoma from radium so screened that the tissues received mainly, but not quite exclusively, γ-rays. She found that exposures of at least ten hours were required to produce a regular response. With periods of irradiation of ten hours, respiration, but not aerobic glycolysis, was depressed. When eighteen hours' exposure was employed both respiration and aerobic glycolysis were depressed. A similar result was obtained with normal animal tissues. Krontowski [1933], using a similar source of radiation, found that an exposure sufficient to produce delayed death of his tissue cultures was insufficient to impair glycolysis. Krontowski was, however, studying effects subsequent to irradiation. Holmes [1933] studied the effect of γ-radiation upon the metabolism of embryonic kidney tissue. An exposure of 14 hours was allowed and the culture fluid analysed after two days' development of the culture. Thus the effects here measured were effects subsequent to irradiation combined with the effects contemporaneous with the irradiation. This treatment resulted in a 50–60 % inhibition of carbohydrate breakdown but had no effect upon protein breakdown. With smaller doses results suggestive of stimulation of carbohydrate breakdown were obtained.

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[1] Beit Memorial Research Fellow.

EXPERIMENTAL.

Every irradiated culture was studied simultaneously with normal control cultures taken from the same freshly inoculated material as the irradiated culture and differing from it only in as much as they were at no time exposed to γ-radiation. Thus a typical experiment carried out to observe the effect of irradiation from the 8th to the 11th hour would involve the following observations. After eight hours' growth one Petri dish would be taken for counting and estimation of ammonia content. A second Petri dish would be placed on radium at this time. At eleven hours this culture on radium and also a second normal control culture would be taken for counting and analysis. Thus the gain in numbers and ammonia content could be found for (*a*) a normal culture developing from the 8th to the 11th hour and (*b*) for an exactly similar culture undergoing irradiation during this period. The assumption, which other experiments have proved to be justified, is made, that the culture set to develop on radium is, at the 8th hour, very nearly identical with the other normal cultures.

The γ-radiation was applied from the following source. The radium element was enclosed in a soldered silver plaque. It amounted to 400 mg. divided over an area of 38·5 cm.^{2} and was contained in cells of 0·1 mm. thickness of platinum further screened with 1·0 mm. of silver. The plaque may be considered as representing a concentration of 10·38 mg. per cm.^{2}, and the distance of the bodos from the radium was 0·5 to 0·75 cm.

DISCUSSION OF RESULTS.

Our results are summarised in Table I.

The unit of area employed in measuring the cells is 0·25 micron^2.

In the first column of the table the periods during which irradiations were carried out and the age of the culture when these irradiations were applied are tabulated. The irradiated bodos were invariably larger than the bodos in the corresponding normal control cultures. In the third column, the effects of the irradiations upon the total number of cells in the cultures are given. In each case the figures express the number of bodos in the irradiated culture per 100 bodos in the normal culture. In the fourth column, the figures for the increase in the number of bodos in the normal and irradiated cultures during the period of irradiation are presented in the same way. The morphological changes produced by the irradiation are summarised in the fifth column. A classification of the periods of irradiation on a rather broader basis than that used in the first column is given in the sixth column, and the influence of these irradiations upon the rate of ammonia production is tabulated in the seventh column. The method by which these figures, termed here the "ammonia ratio," were obtained is as follows. The ammonia produced during any given period was measured in both irradiated and control cultures. This quantity of ammonia was divided by the number of bodos present in the culture at the end of the period. This ratio represents the nitrogenous metabolism, calculated on a "per bodo" basis, associated with the development of the culture from an initial state, common to both irradiated culture and control, to the final state found in the culture at the end of the period of observation. Finally, this ratio, calculated for the irradiated

Table I. *The effect of γ-ray irradiations of different durations upon the size, numbers and ammonia production of the cells in actively growing cultures of* Bodo caudatus.

I	II	III	IV	V	VI	VII
Duration of exposure	Disparity in the mean cell size of irradiated compared with normal cultures at the end of the period of exposure Units of area	Average discrepancy in total numbers present at end of exposure Normal/Irrad.	Average ratio of increase in numbers Normal/Irrad.	Nature of cell response in the irradiated culture	Duration of exposure	Ammonia ratio
2 hours, from 5th to 7th hour	4·35	100 : 95·7	100 : 80·0	Slight check in division. No significant rise in the size of the cells. Slight response Check in division, with gradual increase in the mean size of the cells and in their size at the moment of division	2–4 hours, between 5th and 14th hours	30 % decrease
3 or 4 hours, between 5th and 14th hours	9·03	100 : 76·2	100 : 50·0			
5 hours from start	14·55	100 : 65·1	100 : 25·4	Marked forcing up of the mean size of the cells. Check in division, merging gradually into acceleration of division	5–9 hours, between start and 9th hour	102 % increase
7½ and 9 hours from start	14·33	100 : 72·5	100 : 50·3			
15 hours, from 7th to 22nd hour	12·65	100 : 65·6	—	Maintenance of the enhanced size of the cells and the establishment of a rate of multiplication not far removed from the normal	15–22 hours	42 % increase
22 hours from start	18·52	100 : 62·2	100 : 56·2			

culture, is expressed as a percentage increase above, or decrease below, the same ratio calculated for the normal control culture. It is these percentages which appear in column VII.

It is seen that γ-irradiation can produce very different effects upon the nitrogenous metabolism according to the duration of the exposure and its morphological effects. Thus, progressively increasing periods of irradiation give first an inhibition in the rate of ammonia production per bodo, then a marked increase and finally a less marked increase. The inhibition of ammonia production coincides with the least marked effects upon the size and rate of division of the cells. The greatest stimulation of ammonia production, on the other hand, coincides roughly with the most marked effects upon the size and rate of division of the cells. Finally, an effect upon the ammonia production of an intermediate degree—*i.e.* a moderate stimulation—coincides with a well-marked effect upon the size of the cells, appearing in the absence of any marked effect upon their rate of division. This last morphological effect was well illustrated in the graph showing the growth rates of normal and irradiated cultures published in 1932 [Robertson, 1932]. The resumption of the normal rate of division and fall in the "ammonia ratio" coincided with the development of the second generation of cells produced in the presence of the radium. It would seem that the capacity for the resumption of growth at this normal rate in irradiated cultures is connected with, or conditioned by, the physiological and morphological changes taking place during the period of the greatest stimulation of ammonia production, which is also the period of the evolution of the first generation of cells exposed to the γ-rays.

It is important to note that the same type of effect is produced upon the total ammonia production of the cultures by the different durations of irradiation described above as is produced upon the ammonia production per bodo. Thus the increased value of the "ammonia ratio" in the longer irradiations is not merely an expression of the fact that there are fewer and larger bodos in the irradiated cultures.

We have no means of deciding from our data as to whether the effects upon ammonia production are secondary to the morphological effects, or *vice versa*, or whether all these effects are directly produced by the γ-radiation.

The qualitative contrast between the effects of very small and of large doses of γ-radiation is, of course, in accordance with the biological effects of many toxic agents.

SUMMARY.

Cultures of the protozoon *Bodo caudatus* have been exposed to γ-radiation. The effects of varying the duration of this exposure upon the cell size, rate of multiplication and the rate of ammonia production in the culture are described.

We are indebted to the Radium Committee of the Medical Research Council for kindly supplying the radium used in this research.

REFERENCES.

Crabtree (1932). *10th Sci. Rep. Imp. Cancer Res. Fund*, 33.
Holmes (1933). *Biochem. J.* **27**, 391.
Krontowski (1933). *Arch. exp. Zellforsch.* **13**, 407.
Lawrie (1935). *Biochem. J.* **29**, 547.
Robertson (1932). *Quart. J. Micr. Sci.* **75**, 511.
—— (1935). *Brit. J. Radiol.* **8** (in press).

CXXV. NOTE ON THE SPONTANEOUS DECOMPOSITION OF LECITHIN.

By CHRISTOPHER WILLIAMS PRICE.
47, Blackmores Grove, Teddington, Middlesex.

(*Received March 21st, 1935.*)

The most recent work on the decomposition of lecithin is due to Fischgold and Chain [1934], who have obtained evidence that lecithin, even in the absence of air, undergoes a spontaneous change. This change was found to be due to separation of free fatty acid from the lecithin molecule.

C. W. Price and Lewis [1933, 1] noted a change in the electrophoretic properties of lecithin as it became aged even though the material was kept in the dark and in an atmosphere of dry nitrogen. In the p_H region in which the lecithin dispersion was negatively charged, it was found that there was a rapid increase in electrophoretic mobility with the age of the lecithin used for the dispersion. After a period of about 3 weeks the lecithin reached some state of equilibrium, its electrophoretic properties remaining constant (at a given p_H) for at least 5 months.

Fig. 1 shows the effect of age on the mobility of lecithin at p_H 5·02 and Fig. 2 compares the mobility-p_H curve of lecithin in its stable condition (*A*), with that of lecithin aged for 8 days only (*B*). Owing to the continuous change in the behaviour of the substance, the latter curve is approximate only. However, the points were plotted in such an order that, as nearly as possible, the curve is representative of lecithin aged for the above period.

The lecithin was prepared from egg-yolk by the method of Levene and Rolf [1927] and the measurements were carried out by the macro-electrophoretic method described by C. W. Price and Lewis [1933, 1].

From the curves it is evident that there is a very large change in the electrophoretic properties of lecithin with age, including a shift in the isoelectric point towards the acid region.

The isoelectric point of lecithin in the "aged" or equilibrium condition has been found by C. W. Price and Lewis to be at p_H 2·7. This corresponds fairly closely to the values obtained by other methods employed by workers in this field. Thus Fugii [1924] observed the isoelectric point of lecithin to be at p_H 2·7, H. I. Price and Lewis [1929] at p_H 2·6 and Remezov [1930] at p_H 2·0–2·8. It might be concluded therefore, that the material used by these authors was similar to the "aged" material used by us. Recently Chain and Kemp [1934] have reported the isoelectric point of freshly prepared lecithin as p_H 6·7; a value more nearly approximating to that calculated, namely p_H 7·5[1].

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[1] This value was estimated from the values of the dissociation constants of choline and of glycerophosphoric acid (and similar phosphoric acid derivatives) and is a correction of the value (p_H 5·06) estimated in the same way by C. W. Price and Lewis but based on an erroneous value of K_b for choline. This correction is acknowledged; the error being consequent upon the assumption that the reproducibility of K_b, calculated from the p_H of an extreme range of concentrations of choline chloride, was sufficient evidence to show the absence of traces of free acid or free base. This error was likewise observed by Jukes [1934].

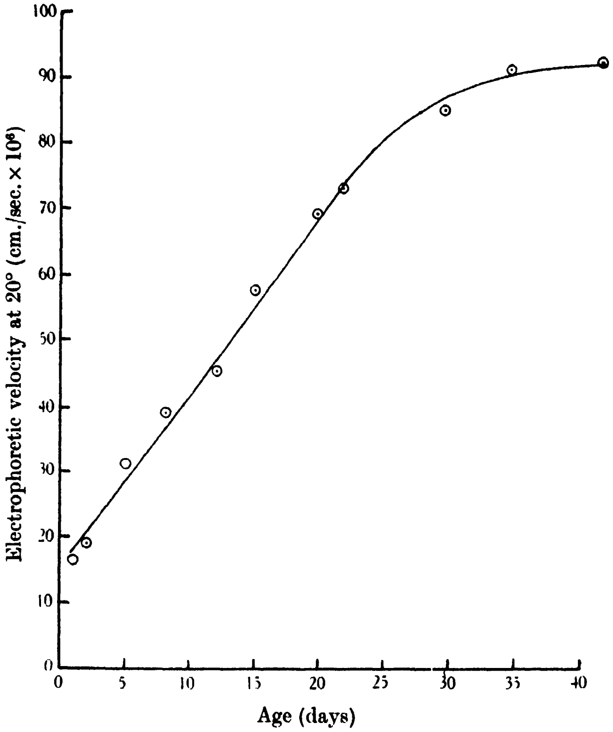
Electrophoretic velocity at 20° (cm./sec. × 10⁶)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Age (days)

Fig. 1.

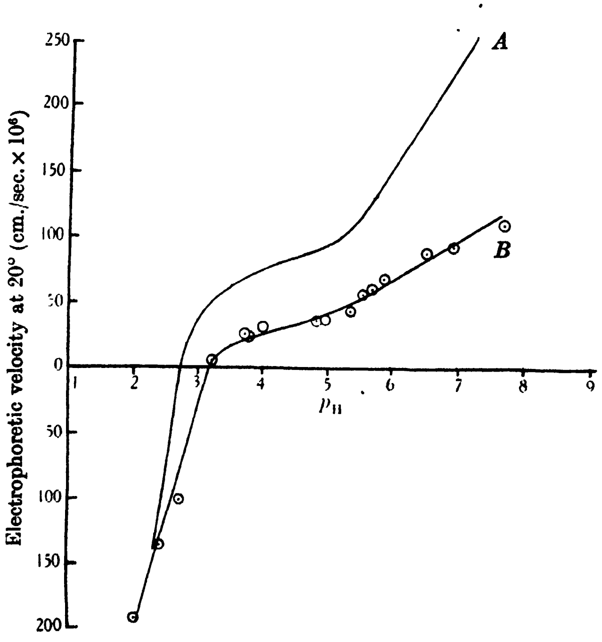
Electrophoretic velocity at 20° (cm./sec. × 10⁶)
250
200
150
100
50
0
50
100
150
200
1
2
3
4
5
6
7
8
9
pH
A
B

Fig. 2.

As previously stated, Fischgold and Chain have shown that the spontaneous change undergone by lecithin in the absence of air, is due to the separation of free fatty acid. From a knowledge of this Chain and Kemp [1934] consider, and in the writer's view quite properly, that the low value of the isoelectric p_H obtained by earlier workers is due to the presence of this material. The reason for this peculiar effect of free fatty acid in a buffered solution is, however, not considered. Clearly, the presence of free fatty acid as such, can influence the electrophoresis of the dispersion only if it is adsorbed at the surfaces of the lecithin micelle. Consequently this becomes, simply, a special case of ion adsorption, *viz.* the adsorption of fatty acid anions (or molecules subsequently ionising). This is in accordance with the view expressed by C. W. Price and Lewis [1933, 1, 2] that the charge on "aged" lecithin is a composite factor, involving an adsorption charge in addition to the ionisation charge. The source of the adsorption charge is the free fatty acid anions rather than simple OH^- ions as tentatively suggested. The reality of an adsorption charge was demonstrated by C. W. Price and Lewis [1933, 2] from determinations of the isoelectric point of the lecithin in dispersions of increasing ionic strength. It was shown that a very large shift in the isoelectric p_H from p_H 2·7 to 5·1 was consequent upon the addition of small concentrations (*e.g.* 0·03 *M*) of $BaCl_2$. Further increase in the ionic strength of the solution merely resulted in the mobility-p_H curve revolving around this point, as would be expected if, at p_H 5·1, the adsorption charge was completely nullified. The fact that the isoelectric point did not shift above p_H 5·1 to a value nearer to that calculated would seem to indicate the presence of some very small quantity (about 10^{-2} mol. %[1]) of further ionising groups in the molecule formed by the inevitable slight oxidation of the lecithin. This is probably also the explanation of the discrepancy between the calculated isoelectric point and that observed by Chain and Kemp (p_H 6·7) for freshly extracted lecithin, rather than the presence of a very small quantity of free fatty acid suggested, since no shift was observed in the isoelectric point on varying the ionic strength of the dispersion. This criterion was used by Chain and Kemp to show the absence of an adsorption charge on the fresh material.

The susceptibility of lecithin for acquiring an adsorption charge can be seen from the fact that at fairly high concentrations of $BaCl_2$ (*e.g.* 0·15 *M*) it becomes charged positively at all p_H values[2].

It should be stressed that in the experiments of the writers only "aged" lecithin was used. It is quite probable that lecithin *in situ* resembles this equilibrium material more closely than that freshly extracted. Thus Fischgold and Chain [1934] point out that by precipitation of the lecithin with cadmium chloride (a process used in its purification) it is possible to remove the free fatty acid.

Since the physiological function of certain phosphatides is usually associated, at least in part, with their great dispersing properties, the fact that freshly extracted lecithin emulsifies only with difficulty (a phenomenon also observed by Fischgold and Chain) gives some support to the above view.

Again, the opacity of a lecithin dispersion decreases very markedly with the age of the lecithin used, until the equilibrium position is reached. This would be expected if the material becomes more hydrophilic on ageing, the refractive index of the material approaching more closely to that of water. The increase in hydrophilic nature might well be associated with the increased charge (of a negative sign) due to the presence of fatty acid adsorbed on the lecithin micelle. The process of purification would seem, therefore, to decrease, at least temporarily, the dispersing qualities of the lecithin by removing the free fatty acid and also as a consequence of this, to render it less hydrophilic.

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[1] The ionisation is assumed to be from carboxyl groups formed by oxidation of unsaturated glycerides with a p_K value of 5.

[2] This phenomenon was observed for the fresh material by Chain and Kemp [1934] and for the "aged" material by C. W. Price and Lewis [1933, 2].

SUMMARY.

The spontaneous change taking place in lecithin, in the absence of air, has been shown to be associated with a change in its electrophoretic properties (resulting in an increased charge of negative sign) and a decrease in the isoelectric p_H.

It is considered that lecithin aged to constant electrophoretic mobility in the absence of air, more closely resembles the state of the material *in situ* than does the freshly extracted substance, since free fatty acid present is removed in the process of purification.

REFERENCES.

Chain and Kemp (1934). *Biochem. J.* **28**, 2052.
Fischgold and Chain (1934). *Biochem. J.* **28**, 2044.
Fugii (1924). *J. Biochem.* **3**, 393.
Jukes (1934). *J. Biol. Chem.* **107**, 783.
Levene and Rolf (1927). *J. Biol. Chem.* **72**, 587.
Price, C. W. and Lewis (1933, 1). *Trans. Faraday Soc.* **29**, 774.
— — —— (1933, 2). *Trans. Faraday Soc.* **29**, 1187.
Price, H. I. and Lewis (1929). *Biochem. J.* **23**, 1030.
Remezov (1930). *Biochem. Z.* **218**, 86.

CXXVI. A NOTE ON THE NITROGEN OF THE POLYSACCHARIDE COMPLEX FROM *CHONDRUS CRISPUS*.

BY MARGARET RUTH BUTLER.

From the Department of Biology, Dalhousie University, Halifax, Nova Scotia.

(*Received March 16th, 1935.*)

DURING the investigation of some biochemical properties of the polysaccharide extract of the alga, *Chondrus crispus*, varying quantities of nitrogen were found in different samples. The amount of nitrogen present in the extracts as prepared by a standard method [Butler, 1934] was found to differ from one collection of plants to another. In all samples prepared from the same collection of plants, however, it was constant. This indicated that the variations of nitrogen in the extracts depended on variations in the samples from which they were prepared. The total nitrogen was, therefore, determined in a number of collections and in the standard extracts from these same collections. The results (Table I) show that the differences occurring from one collection of plants to another are great; also that in general the nitrogen content of the extract varies as that of the collection from which it is made.

Table I. *Percentage of nitrogen in plants and extracts.*

Sample	% nitrogen in plants (dry weight)	% nitrogen in extract (dry weight)
S	0·74	0·26
N	1·75	0·39
H*	4·05	2·75
C	0·99	0·24
B	0·93	0·29

* It is interesting that Sample H, which shows the greatest deviation from the average nitrogen content, is the only one collected during the winter. Though this is but an incidental observation, it has suggested further investigations, now being carried on regarding seasonal variations.

In an attempt to purify the polysaccharide extract by reprecipitation, the effect on its nitrogen content was noted. As shown in Table II, the change effected was negligible.

Table II. *Percentage of nitrogen in re-precipitated extracts.*

Sample	% nitrogen in extract (dry weight)	% nitrogen in re-precipitated extract (dry weight)
T	0·85	0·86
P	1·21	1·10
D	1·43	1·18
S	0·26	0·30
F	3·55	2·25
M	0·81	0·62
B	0·28	0·29
C	0·24	0·24
C*	0·27	0·27

* 3 times precipitated.

In order to obtain some idea of how the nitrogen is distributed in the plant, it has been determined, as % of dry weight, in various extracts and residues (Table III).

Table III. *Percentage of nitrogen in fractions of* Chondrus.

Sample	% nitrogen in dry weight
Whole plant	0·93
Aqueous extract (not precipitated)	0·64
Residue from aqueous extract	2·06
Crude precipitate	0·28
Alcohol-soluble fraction of aqueous extract	1·13
Purified precipitate (Standard Extract)	0·29
Residue of plants freed from all water-soluble constituents	3·85
Alcoholic extract	0·71
Residue from alcoholic extract	0·82

The highest percentage of nitrogen per dry weight is found in the residue from plants washed under running water for a week or ten days. This is doubtless due to the great decrease in carbohydrate which makes this fraction itself small. Thus, while the percentage of nitrogen per dry weight is high, expressed as % of total nitrogen it would be very small. Because of the colloidal and highly viscous nature of the extract, the difficulties involved in manipulation are great. For this reason it is impossible to make a truly quantitative fractionation of the plants, but an approximation is given in Table IV.

Table IV. *Fractionation of plants.*

Fraction	% whole plant
Aqueous extract	65–67
Residue from above	33–35
Alcohol precipitate of aqueous extract	50–54
Alcohol-soluble aqueous extract	13–15

In Table V is given the percentage distribution of nitrogen as calculated by combining Tables III and IV.

Table V. *Percentage distribution of nitrogen in plant fractions.*

Fraction	% total nitrogen
Whole plant	100
Aqueous extract	37
Residue from above	63
Alcohol precipitate of aqueous extract	13
Alcohol-soluble aqueous extract	15

Identification of samples.

Sample	Place of collection	Month of collection
S	South Shore, P.E.I.	August
N	Souris, P.E.I.	August
H	Halifax, N.S. (Pt. Pleasant)	February
T	Halifax, N.S. (McNab's Island)	August
D	Halifax, N.S. (Devil's Island)	June
P	Peggy's Cove, N.S.	August
C	Cavendish, P.E.I.	August
B	Borden, P.E.I.	August
F	Details of collection missing. Extract made without any washes of absolute alcohol	—
M	Mixture of extracts from different samples dissolved together and precipitated	—

DISCUSSION.

If the nitrogen were simply an impurity one would expect that it might be removed, or at least appreciably reduced, by repeated precipitations. There is, however, no indication of such reduction. On the other hand, there is no evidence that the nitrogen content can be brought to a constant figure for all samples, as might be expected if it were chemically united with the polysaccharide complex (Table II).

No doubt there is protein material in the plants, but in the extract from only one sample, *viz.* H, could a positive biuret test be obtained. The protein nature of the nitrogen in the polysaccharide extracts, therefore, seems doubtful. Furthermore, no nitrogen was detected by the Van Slyke amino-nitrogen method.

Haas [1921] and Russell-Wells [1922] reported positive protein tests for their extracts of *Chondrus*. More recently, Haas and Hill [1933] have reported a sample of *Chondrus* with a total nitrogen content of 2·33 %, as giving a negative biuret test.

Russell-Wells [1922] found that nearly half of the 0·56 % of nitrogen in the extract she used occurred in an ammonium radical. In the present investigation none was identified as such.

From a consideration of these results no suggestion can be offered as to the form in which the nitrogen present exists. It is possible, however, that the nitrogen which accompanies the carbohydrate complex through repeated precipitations does so because it is adsorbed on the colloidal particles.

SUMMARY.

The percentage of nitrogen in the polysaccharide complex extracted from *Chondrus crispus* by a standard method is found to depend on the amount of nitrogen originally present in the plants.

The percentage of nitrogen in this complex is not reduced by repeated precipitations.

The amount of nitrogen associated with this carbohydrate material is calculated to be approximately 13 % of the total nitrogen in the plants.

The nature of this nitrogen is still undetermined.

REFERENCES.

Butler (1934). *Biochem. J.* **28**, 759.
Haas (1921). *Biochem. J.* **15**, 469.
—— and Hill (1933). *Ann. Bot.* **47**, 55.
Russell-Wells (1922). *Biochem. J.* **16**, 578.

CXXVII. THE CATALYTIC OXIDATION OF ASCORBIC ACID.

By ALEXANDER EDWARDS KELLIE
AND SYLVESTER SOLOMON ZILVA[1].

From the Division of Nutrition, Lister Institute, London.

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As long ago as 1922, Hess [1922] demonstrated the possibility of destruction of vitamin C through the catalytic action of metals. He showed in this connection that when milk was pasteurised in glass containers it was antiscorbutically more active than when it was submitted to this treatment in copper vessels. Recently, Euler *et al.* [1933] found that pure ascorbic acid took up oxygen more readily in the presence of certain metals. The experiments described in this communication, which were the outcome of an observation on the instability of ascorbic acid in distilled water, yield further information on this subject.

The rate of oxidation of ascorbic acid dissolved in water of different degrees of purity.

In these experiments the spontaneous oxidation of ascorbic acid in tap water, ordinary laboratory distilled water obtained from a tin-lined copper still, water redistilled from glass apparatus and water distilled from and received in quartz apparatus was investigated. 15 mg. of *l*-ascorbic acid were dissolved in 40 ml. of each sample of water contained in conical flasks of 100 ml. capacity. In the case of the quartz-distilled water round-bottomed flasks of 50 ml. capacity were used. The reaction in each case was adjusted to p_H 7·4 by addition of the requisite quantity of sodium hydroxide and the flasks were kept at 37°. At the end of 20 hours the reaction in each case fell to about p_H 6·7. 5 ml. of the solutions were withdrawn at various intervals and the ascorbic acid was determined by titration with indophenol. The results are given in Table I, from which it will be seen that the rate of disappearance of ascorbic acid in tap water and in laboratory distilled water was fairly quick but that the oxidation in glass-distilled and quartz-distilled water was very much slower. It should be mentioned, however, that unless the distillation of the water in glass was carried out three times and the product used immediately the low rate of oxidation was not observed. On the other hand, when oxygen was bubbled for one hour through

Table I.

mg. of ascorbic acid per 100 ml. of water.

Hours	Tap	Ordinary distilled	Glass-distilled	Quartz-distilled
0	35·0	35·4	37·7	37·9
1	25·6	25·8	37·6	37·5
2	15·1	15·2	37·5	37·2
3	6·9	6·3	36·8	37·0
4	1·4	1·9	36·3	36·7
20	0·0	0·0	25·6	31·6

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[1] Member of the Scientific Staff, Medical Research Council.

this water the rate of oxidation of the vitamin was not significantly accelerated. These results suggested that the increase in the rate of oxidation of ascorbic acid in tap water or in ordinary distilled water was due to the presence of traces of metal. Subsequent experiments proved this view to be true.

The catalytic action of traces of metals present in ordinary distilled water on the oxidation of ascorbic acid.

The first step taken in order to ascertain whether the higher rate of oxidation of ascorbic acid in tap water and ordinary distilled water was due to the catalytic effect of substances dissolved in the water, was to establish whether the addition of such substances to glass-distilled water would accelerate the oxidation of ascorbic acid. For this purpose the incinerated and untreated residues left on evaporation of ordinary distilled water were employed. These were obtained in the following way. Ten litres of ordinary distilled water were evaporated to about 100 ml. The liquid was then divided into two equal quantities, which were evaporated to dryness on a water-bath; one of the residues was then incinerated. In each case the residue was taken up in 100 ml. of glass-distilled water and the oxidation of ascorbic acid dissolved in these two samples was determined by the procedure mentioned above. These results which are given in Table II show that both residues accelerated the oxidation.

Table II.

mg. of ascorbic acid per 100 ml. of water.

Hours	Glass-distilled	Glass-distilled + untreated residue	Glass-distilled + incinerated residue
0	37·9	36·7	35·1
¼	36·8	28·6	24·2
1½	34·9	19·8	13·7
3	33·3	8·8	6·3
20	26·5	0·0	0·0

In view of the fact that the inorganic residue increased the rate of oxidation in glass-distilled water an experiment was performed in which cyanide was added to the ordinary distilled water. The results (Table III) showed that the rate of disappearance of the ascorbic acid fell to about the same level as in glass-distilled water thus suggesting that at least some of the inorganic constituents contained in the normal sample were catalysing the oxidation. This water was therefore submitted to an examination for iron and copper, since it has been shown by Euler *et al.* [1933] that traces of these metals, especially of the latter, were capable of catalysing the absorption of oxygen by ascorbic acid in a Barcroft apparatus. The presence of such traces could well be expected in

Table III.

mg. of ascorbic acid per 100 ml. of water.

Hours	Ordinary distilled	*M*/25,000 sodium cyanide in ordinary distilled
0	33·0	37·0
1	23·5	36·7
2	18·1	36·1
3	13·7	35·3
4	11·1	34·6
20	0·0	27·4

distilled water obtained from a laboratory still. The detection of iron was carried out with analytically pure potassium thiocyanate according to Sakuma [1923]. The reaction was found to be positive in water concentrated 200 times, the concentration of the iron being, however, too low for quantitative determination. After precipitating the iron with ammonium hydroxide the copper was determined colorimetrically with sodium diethyldithiocarbamate and was found to be present in a concentration of 0·001 mg. per 100 ml. of water.

It was then possible to demonstrate that when these metals were added to quartz-distilled water the oxidation of ascorbic acid proceeded at an increased rate (Table IV). This was particularly marked in the case of the added copper which is in consonance with the results of Euler *et al.* [1933]. It may be mentioned that when quantities of copper were added to bring up the concentration to that found in the ordinary distilled water, the rate of oxidation was also raised but was less than that observed in the above experiment.

Table IV.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + 0·01 mg. Fe/40 ml.	Quartz-distilled + 0·01 mg. Cu/40 ml.
0	37·0	34·5	34·1
1	35·2	31·8	24·2
2	33·0	29·6	21·2
3	30·9	28·2	15·2
4	29·4	25·8	8·0
20	25·7	10·3	0·0

The next experiment was instituted with the object of establishing whether by treating ordinary distilled water by the usual procedures employed in the removal of the bulk of dissolved oxygen the rate of oxidation of the vitamin could be significantly modified. The results obtained (Table V) showed that with the quantities of ascorbic acid used, this was not the case. Furthermore, as already mentioned, when the catalysts are absent, the water (fresh glass-distilled)

Table V.

mg. of ascorbic acid per 100 ml. of water.

Hours	Ordinary distilled	Ordinary distilled. Air removed by boiling	Ordinary distilled. Air removed by evacuation	Ordinary distilled. Air replaced by passing nitrogen
0	34·9	34·7	33·0	34·4
½	28·4	30·2	28·8	30·2
1½	15·8	16·5	18·1	17·2
3	5·4	8·1	10·4	4·7
5	0·4	2·5	4·7	1·6
20	0·0	0·0	0·0	0·0

Table VI.

mg. of ascorbic acid per 100 ml. of water.

Hours	Glass-distilled	Glass-distilled saturated with air	Glass-distilled saturated with oxygen
0	37·7	36·4	36·6
½	37·6	36·2	36·5
1½	37·0	35·1	35·2
3	36·3	34·3	34·1
5	35·8	33·3	33·2
20	27·4	24·4	23·9

may be saturated by previously passing through it a current of air or oxygen without significantly accelerating the disappearance of the dissolved ascorbic acid (Table VI).

The inhibiting effect of animal tissues and other substances on the oxidation of ascorbic acid in ordinary distilled water.

It has recently been observed [Quastel and Wheatley, 1934; De Caro and Giani, 1934; Mawson, 1934] that when animal tissues or their extracts were added to a solution of ascorbic acid the oxidation of the vitamin was greatly inhibited. In other words the action of the tissue is similar to that of cyanide in this connection. Similar observations had already been made in this laboratory independently, but as a detailed investigation on this subject has already been published by De Caro representative experiments only will be reported here for the purpose of continuity.

The technique employed in these experiments was as follows. One g. of tissue (guinea-pig) was thoroughly ground with 10 parts by weight of ordinary distilled water. After centrifuging, the supernatant liquid was added to 30 ml. of an aqueous solution containing 15 mg. of ascorbic acid thus bringing up the total volume to 40 ml. The incubation and determination of the ascorbic acid were carried out as above. The results of such an experiment are given in Table VII from which the stabilising action of tissue extracts becomes evident. Extracts from the large and the small intestine were found by us also to be effective. Like De Caro, we found that extracts from the tissues of scorbutic animals exercised the same effects as those from tissues of normal guinea-pigs.

Table VII.

mg. ascorbic acid per 100 ml. of water.

Hours	Liver	Kidney	Muscle	Spleen	Plasma	Ordinary distilled water
0	38·2	37·5	37·0	37·5	36·8	32·0
1	37·7	36·3	36·1	37·4	36·1	24·9
2	37·4	35·6	35·1	37·0	35·4	15·2
3	37·0	34·7	34·6	36·7	35·2	9·8
4	36·8	34·2	33·7	36·3	34·6	5·3
20	31·1	25·6	28·8	23·5	24·0	0·0

That the inhibiting action of animal tissues and their extracts was due to their influence on the catalytic action of metals present in solution was proved by the following experiments in which graded amounts of iron and copper were added to solutions containing ascorbic acid and liver tissue extracts prepared as above. The oxidised ascorbic acid was determined in the usual way. The results (Table VIII) showed that although copper is more effective as a catalyst

Table VIII.

mg. of ascorbic acid per 100 ml. of water.

Hours	Tissue extract	Tissue extract +0·01 mg. Fe/ 40 ml.	Tissue extract +0·02 mg. Fe/ 40 ml.	Tissue extract +0·05 mg. Fe/ 40 ml.	Tissue extract +0·10 mg. Fe/ 40 ml.	Tissue extract +0·05 mg. Cu/ 40 ml.	Tissue extract +0·10 mg. Cu/ 40 ml.	Tissue extract +0·20 mg. Cu/ 40 ml.	Tissue extract +0·30 mg. Cu/ 40 ml.	Tissue extract +0·50 mg. Cu/ 40 ml.
0	39·3	39·3	39·5	39·8	39·1	39·1	39·5	39·7	39·5	36·7
1	39·1	39·1	38·8	37·0	35·3	39·0	39·3	39·5	39·1	29·8
2	39·0	39·0	37·7	34·4	32·4	38·6	39·1	39·0	37·0	25·1
3	38·8	38·7	37·2	32·4	29·5	37·9	38·4	38·4	35·4	20·4
4	37·7	37·7	35·8	30·7	26·4	37·7	37·7	36·5	33·2	15·4
20	33·2	31·2	24·7	12·6	4·6	32·4	32·1	24·9	6·0	1·4

than iron in this process, the inhibiting action of the tissue extract is very much more marked in the former case. It should be mentioned here that ordinary distilled water which contains copper and iron was used in these experiments and consequently the actual amount of catalyst put out of action was rather higher than that suggested by the figures in Table VIII.

De Caro observed further the interesting fact that Ringer's solution and the salts of which it is made up had also a stabilising effect on ascorbic acid. We have repeated this part of his work since the appearance of his paper and have been able to confirm his results. The protective action of the salts will be seen from the results given in Table IX. It was of interest to ascertain whether this protective action was, as in the case of animal tissues, due to the suppression of the catalytic action of metals. The rate of oxidation was therefore studied in the presence and in the absence of NaCl in quartz-distilled water to which copper was added. The results obtained were clear cut and as will be seen from Table X the oxidation catalysed by Cu was very much slower when NaCl was present. It may be noted that the addition of N/10 NaCl did not reduce the velocity of the reaction to that of the control solutions of ascorbic acid which did not contain any copper.

Table IX.

Hours	mg. of ascorbic acid per 100 ml. of water. N/10 KCl	N/10 NaCl	N/10 $CaCl_2$	Ringer solution	Ordinary distilled water
0	35·4	33·3	32·8	31·8	35·6
1	32·8	30·0	30·6	27·0	27·0
2	30·5	27·0	26·8	23·0	16·4
3	28·6	25·6	24·2	17·7	6·8
4	26·7	22·8	21·1	14·6	1·4

Table X.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + N/10 NaCl	Quartz-distilled + N/10 NaCl + 0·01 mg. Cu/40 ml.	Quartz-distilled + 0·01 mg. Cu/40 ml.
0	38·6	37·4	37·7	35·4
1	38·1	35·8	31·8	28·6
2	37·4	33·9	28·6	21·2
3	35·4	32·8	26·1	15·6
4	33·5	32·3	22·8	7·7

The influence of blood on the catalytic oxidation of ascorbic acid.

Particular attention has been given to this point owing to the fact that it is claimed that in the blood vitamin C is present as dehydroascorbic acid [Emmerie *et al.*, 1933]. The effects of plasma, intact and haemolysed red corpuscles and leucocytes were therefore investigated. It was mentioned above (Table VII) that plasma had an inhibiting action on the catalytic oxidation of ascorbic acid. In the case of the red corpuscles an equivalent of 1 ml. and in that of the leucocytes an approximate equivalent of 10 ml. of blood was added to the same quantity of ascorbic acid and under the same conditions as mentioned above. In both cases the cells were washed three times with an isotonic solution of sodium citrate. It will be seen from Table XI that whilst the erythrocytes offered very marked protection, the leucocytes had no perceptible influence on the rate of oxidation of the ascorbic acid.

Table XI.

mg. of ascorbic acid per 100 ml. of water.

Hours	Intact red corpuscles	Leucocytes	Citrate control	Laked red corpuscles	Ordinary distilled
0	34·2	33·9	34·7	30·7	32·3
1	33·0	28·2	28·9	30·4	23·0
2	31·9	21·9	22·1	30·2	16·7
3	31·2	16·3	17·2	27·4	7·9
4	30·5	11·1	11·6	20·7	3·3

Laked red corpuscles were also found to be capable of protecting ascorbic acid from oxidation but in this case an initial disappearance of the vitamin was noted. It was possible to show that the quantity of ascorbic acid which disappeared was proportional to the amount of haemolysed blood added and further that when the red corpuscles were previously treated with CO the destructive action of the haemolysed erythrocytes was hardly appreciable (Table XII).

Table XII.

mg. of ascorbic acid per 20 ml. of solution determined immediately after addition.

	Control	1 ml.	2 ml.	4 ml.
Intact red corpuscles	7·0	7·1	7·5	7·8
Laked red corpuscles	6·7	5·8	4·9	3·0
Laked red corpuscles treated with carbon monoxide	6·8	6·8	7·0	7·5

It would therefore appear that on haemolysing a substance, most probably oxyhaemoglobin, is set free which is capable of interacting with ascorbic acid.

The product formed by the catalytic oxidation of ascorbic acid.

In the above experiments the disappearance of the ascorbic acid was followed up by titration with indophenol. This procedure did not therefore offer any evidence concerning the character of the oxidation product formed. In the experiments to be described an attempt was made to determine the amount of dehydroascorbic acid present at various intervals, namely after 4, 20, 24 and 28 hours' incubation. The dehydroascorbic acid was determined by titrating an aliquot portion of the solution with indophenol before and after treatment with hydrogen sulphide. The results revealed that after 4 hours' incubation in the cases of both the ordinary distilled water and the quartz-distilled water with added copper a significant part of the vitamin was still present as dehydroascorbic acid although the major part of the ascorbic acid was irreversibly oxidised. There was no dehydroascorbic acid present either in the solution to which cyanide was added or in the quartz-distilled water (Table XIII). After

Table XIII.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + 0·01 mg. Cu/40 ml.	Cyanide	Ordinary distilled
0	38·6	35·4	34·2	36·1
1	38·1	28·6	32·8	28·1
2	37·4	21·2	31·9	14·7
3	35·4	15·6	30·9	7·2
4	33·5	7·7	30·2	2·6
4 hours after treatment with H_2S	34·9	15·4	30·5	7·5

Table XIV.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + 0·01 mg. Cu/40 ml.	Cyanide	Ordinary distilled
0	38·8	36·0	36·7	37·0
4	35·3	8·6	33·3	2·8
20	30·2	0·0	26·7	0·0
20 hours, after treatment with H_2S	30·7	3·7	27·2	3·5
24	29·5	0·0	25·8	0·0
24 hours, after treatment with H_2S	30·0	1·8	26·1	2·1

20 and 24 hours (Table XIV) the quantity of dehydroascorbic acid present in the two above experiments was decidedly less and it was further found that after 28 hours it was only just appreciable in both cases. It would appear therefore that the dehydrogenation of ascorbic acid takes place first and is then followed by irreversible oxidation. This view is further strengthened by the fact that after a short time (about 10 minutes) the loss in the indophenol-reducing capacity of a solution of ascorbic acid can be almost entirely re-established by treatment with hydrogen sulphide.

CONCLUSIONS.

A notable feature in this investigation is the great stability of ascorbic acid in the presence of oxygen when metallic catalysts are absent from solution. The negligible deterioration of the vitamin in quartz-distilled water, previously saturated with oxygen, suggests that in absolutely pure water, ascorbic acid is as stable as in the solid condition; in other words oxygen without the aid of catalysts is inert towards it. In practice, however, it is extremely difficult to produce conditions which would favour such stability of the vitamin in solution. Even the purest distilled water, when allowed to remain in glass vessels for some time, becomes contaminated enough to catalyse the oxidation of ascorbic acid.

It is noteworthy that amongst all the tissues examined only the leucocytes were unable to inhibit the oxidation of ascorbic acid in the presence of catalysts. These cells had nevertheless no accelerating action on the oxidation of the vitamin. There seems, therefore, no indication that ascorbic acid is reversibly oxidised in any part of the animal organism. The observation of Johnson and Zilva [1934] that dehydroascorbic acid is excreted in the urine as ascorbic acid suggests that the tissues on the contrary reduce the reversibly oxidised form of the vitamin. The evidence seems to favour the view that dehydroascorbic acid has to be reduced in the organism before it can function as an antiscorbutic.

SUMMARY.

Ordinary laboratory distilled water contains sufficient quantities of metals (copper and iron) to catalyse the irreversible oxidation of dissolved ascorbic acid. In the very early stages of the process dehydroascorbic acid is found in quantities almost equivalent to the amounts of ascorbic acid which are oxidised, but as the reaction proceeds the quantities of the dehydrogenated acid present diminish. This catalytic action is barely perceptible when the vitamin is dissolved in water which has been redistilled several times from glass apparatus and which is used immediately or in water distilled from and received in quartz. When this water is previously saturated with oxygen the disappearance of ascorbic acid is not significantly increased. The addition of aqueous extracts of

liver, kidney, muscle, spleen, large and small intestine and of plasma, intact or haemolysed erythrocytes and *N*/10 NaCl inhibits the oxidation of ascorbic acid in ordinary distilled water or in water to which iron or copper has been added. Leucocytes have no influence on the oxidation. There is a disappearance of ascorbic acid immediately after the addition of haemolysed erythrocytes which is proportional to the quantity of the added corpuscles. This disappearance does not take place when the haemolysed corpuscles are previously treated with carbon monoxide. It is suggested that dehydroascorbic acid has to be reduced in the organism before it can exercise antiscorbutic activity.

One of us (A. E. K.) is indebted to the Medical Research Council for a whole time grant.

REFERENCES.

De Caro and Giani (1934). *Z. physiol. Chem.* **228**, 13.
Emmerie, van Eekelen, Josephy and Wolff (1933). *Nature*, **132**, 315.
Euler, Myrbäck and Larsson (1933). *Z. physiol. Chem.* **217**, 1.
Hess (1922). *Boston Med. Surg. J.* **187** 101.
Johnson and Zilva (1934). *Biochem. J.* **28**, 1393.
Mawson (1934). *Chem. Ind.* **53**, 1026.
Quastel and Wheatley (1934). *Biochem. J.* **28**, 1014.
Sakuma (1923). *Biochem. Z.* **142**, 68.

CXXVIII. SPECTROPHOTOMETRIC INVESTIGATIONS OF THE NEW VITAMIN A COLOUR TEST.

By EUGENE ROSENTHAL and MARGARET WELTNER.

Budapest, Hungary.

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Colorimetric determination of vitamin A has frequently been based upon the Carr-Price reaction, namely the production of a blue colour when vitamin A is added to a saturated solution of antimony trichloride in chloroform, which shows two absorption maxima at 606 $m\mu$ and at 572 $m\mu$, respectively. This reaction has the following drawbacks: (1) the blue colour is not specific for vitamin A, being given also by carotene, (2) the colour shows a considerable change after the lapse of 30 seconds, rendering colorimetric estimation very difficult. In order to eliminate this difficulty Rosenthal and Erdélyi [1934, 1] devised a new colour test. In this the vitamin-containing material is warmed with a solution of antimony trichloride and catechol in chloroform. The resulting blue colour soon turns into a purplish red. This colour, quite similar to that of a dilute solution of potassium permanganate, is much more stable than the blue colour obtained with the Carr-Price reaction. No other carotenoids except vitamin A produce this colour; the blue colour obtained with other carotenoids does not change on warming. Rosenthal and Erdélyi emphasise the fact that the purplish red hue produced by catechol remains unchanged for some 5 to 10 minutes if fairly pure preparations of vitamin A are used. This fact made possible a more thorough spectrophotometric examination of this purplish red colour.

In our investigations we employed the König-Martens spectrophotometer at a layer depth of 0·2 cm., a Nitra bulb being used as a light source. The extinction coefficient has been calculated according to the following equation:

$$E=\frac{\log tg\alpha_2-\log tg\alpha_1}{d.c},$$

where α_2 and α_1 are the angles measured, d is the layer thickness and c the concentration. A vitamin A concentrate, 0·2 and 0·4 % solution in chloroform has been used.

If reading was performed within the first 10 minutes, two maxima were observed: a broad band at 545 $m\mu$ and a narrower one at 475 $m\mu$. If however the measurement was repeated during the second, third and fourth 10-minute periods, within the first broad maximum two new maxima and a minimum appeared, the latter corresponding to 545 $m\mu$, the maxima corresponding to 535 $m\mu$ and 552 $m\mu$, respectively. At the same time the second maximum also showed a resolution into a lower maximum at 485 $m\mu$ and a considerably higher one at 470 $m\mu$. With the aid of chromatographic analysis Karrer and Morf [1933] recently prepared two fractions of vitamin A, called by the authors vitamin Aα and vitamin Aβ, respectively. Van Eekelen *et al.* [1933] described also a separation of two fractions of vitamin A by fuller's earth. Further research is needed to determine which of these fractions is involved in the production of the above maxima observed by us.

The colour reaction with catechol being more stable is definitely superior to the simple antimony trichloride test; however, the spectroscopic data mentioned above show that the stability even of this colour is not sufficient. This fact induced Rosenthal and Erdélyi [1934, 2] to try to improve and to stabilise their colour test still further. They found that the purplish red hue could be obtained not only with catechol but also with quinol, guaiacol and veratrol. This test therefore can be considered as dependent on the presence of a polyhydric phenol. Other polyhydric phenols however are unsuitable for this test because of their insolubility in absolute chloroform. For quantitative determinations guaiacol has been found optimum among the substances mentioned. A 5 % solution of guaiacol in chloroform has been employed. This modification proved more favourable in several respects: the catechol solution has to be prepared fresh daily, whereas the guaiacol solution may be kept ready for use for days. The final colour of the reaction performed with catechol remains constant only for a short time (5–10 minutes), whereas the final colour of the guaiacol test is quite stable even for 1–2 hours. If very pure preparations are used, the colour does not change even after 24 hours. With the catechol test the fluid becomes turbid after a while, whereas the guaiacol modification has the important advantage in respect of spectrophotometric examination that the fluid remains perfectly transparent.

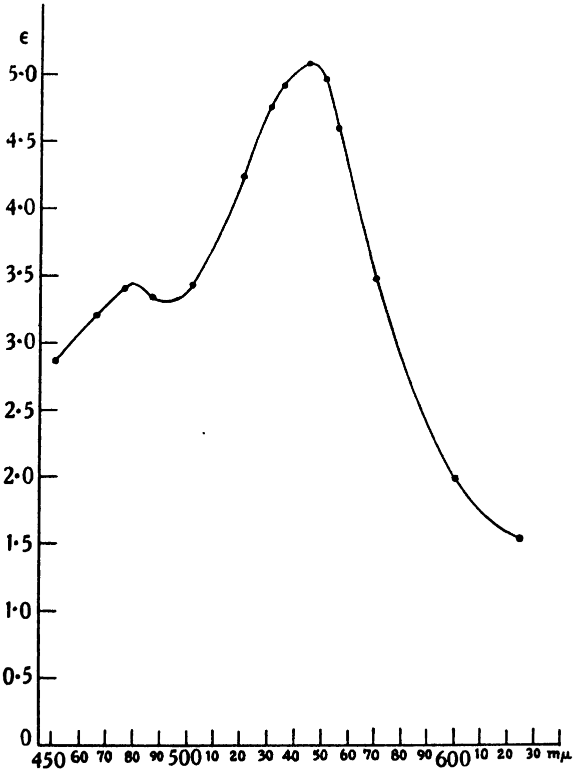

Fig. 1. Absorption curve of colour produced in the antimony trichloride-guaiacol test for vitamin A.

Owing to these circumstances we obtained a quite invariable and characteristic curve when examining the guaiacol reaction spectrophotometrically. It has two maxima, a higher one at 545 $m\mu$, and a lower one at 478 $m\mu$, which are in good accordance with the characteristics of the curve of the catechol test read within the first 10 minutes (see Fig. 1).

As there was no pure vitamin A available, we were unable to determine the quantitative relations of this absorption curve.

SUMMARY.

The purplish red colour of the antimony trichloride-catechol test for vitamin A examined within the first 10 minutes shows two maxima at 545 $m\mu$ and at 475 $m\mu$, respectively. During the second, third and fourth 10-minute periods two other maxima appear.

The purplish red colour of the guaiacol modification of this test remains unchanged for several hours. Spectrophotometric examination of this colour showed two maxima at 545 $m\mu$ and at 478 $m\mu$, respectively.

We are greatly indebted to Prof. J. Gróh, Director of the Chemical Laboratory of the Royal Hungarian Technical College, for valuable suggestions and for providing us with the apparatus needed in our work.

REFERENCES.

van Eekelen, Emmerie, Julius and Wolff (1933). *Nature*, **132**, 171.
Karrer and Morf (1933). *Helv. Chim. Acta*, **16**, 625.
Rosenthal and Erdélyi (1934, 1). *Biochem. J.* **28**, 71.
—— —— (1934, 2). *Biochem. Z.* **271**, 414.

CXXIX. A NEW METHOD OF DETERMINING THE VITAMIN A CONTENT OF BLOOD.

By EUGENE ROSENTHAL and CATHERINE SZILÁRD.

Budapest, Hungary.

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THE first observations relating to the amount of vitamin A in blood are due to Euler and Steffenburg [1928], who stated that the growth of rats kept on a diet devoid of vitamin A was enhanced by adding blood or serum to their ration. Euler also demonstrated that fat extracted from blood serum gives the well-known blue colour reaction with antimony trichloride. The method used for quantitative determination of vitamin A in blood has been published by Rösiö [1929] and consists in a multiple extraction with ether of 25–100 ml. of blood serum, evaporation *in vacuo* of the combined ethereal extracts after drying with sodium sulphate and treatment of the residue with antimony trichloride as described by Carr and Price.

This simple procedure however could not yield accurate results, since not only vitamin A but also carotene is present in variable quantities in blood serum; moreover carotene is extracted by ether, and gives a blue colour with antimony trichloride. Though the growth-stimulating action of carotene upon rats kept on a diet devoid of vitamin A is similar to that of vitamin A, and though in the study of certain questions it may be permissible to determine the quantities of vitamin A and carotene together, it would be fallacious to attribute the results obtained with this method to vitamin A alone, especially since the blue colours produced by allied compounds are of different intensities.

Another method devised by van Eekelen [1931] allows for the presence of carotene by subtraction from the total intensity of the blue colour of that part which is due to carotene. The method of van Eekelen, published later by Menken [1932] and utilised also by Wendt [1935], consists in precipitating 20 ml. of serum with 100 ml. of 96 % alcohol and centrifuging. The supernatant alcoholic fraction is extracted with light petroleum. The slightly yellowish extract is then concentrated *in vacuo* to 1 ml. and utilised for the determination of carotene and later of vitamin A. According to van Eekelen the yellow tinge of this petroleum extract is due solely to carotene, the concentration of which can be determined by colorimetric measurement. The difference between the total blue colour obtained by treatment of the concentrated extract according to Carr and Price and the colour accounted for by the colorimetrically determined carotene is taken to represent vitamin A.

This method of determination seems to be objectionable from several points of view. When determining the carotene content of the blood, Connor [1928] precipitates the serum with 95 % alcohol. After centrifuging, the yellowish alcoholic fraction is discarded on account of its xanthophyll content, whilst the precipitate is extracted with benzene and the carotene content of the serum is determined by colorimetric measurement of this latter extract. Thus the carotene content of the alcoholic fraction and the blue value of carotene to be subtracted finally from the colour of the Carr-Price reaction will vary according to the method of precipitation, the shaking of the tube containing the precipitate and the speed of centrifuging. It must also be remembered that all carotenoids, including vitamin A, are easily adsorbed by different media and it is well-nigh impossible to estimate the amount adsorbed by the protein precipitate. Nor is the yellow colour of the petroleum extract attributable solely to carotene, so that the conversion of the yellow colour into blue units is somewhat uncertain; moreover the reading of the final blue colour must be accomplished within 30 seconds, because of its quick fading. This urgency of reading entails personal error apart from the practical uncertainty of the method, the results of which therefore give at best indirect, and probably often incorrect, information.

These difficulties are overcome by the new colour test of Rosenthal and Erdélyi [1934, 1], which is specific for vitamin A and in which the colour is much more stable, rendering hurried reading unnecessary. The new colour reaction consists in warming the vitamin-containing material with a chloroform solution of catechol or guaiacol [Rosenthal and Erdélyi, 1934, 2] and antimony trichloride, when the initial blue colour of the solution soon turns into a purplish red. This hue remains unchanged for 5 to 10 minutes in the case of catechol and for periods of hours in that of guaiacol, depending on the purity of the material tested. The intensity of the colour obtained is proportional to the concentration of vitamin A and can be measured by means of colorimetric estimation, the colour being identical in tint with that of a dilute solution of potassium permanganate. Since the colour of the permanganate solution changes within a couple of days and the extinction of this changed standard solution does not correspond to that of the coloured solution obtained in the reaction, colorimetric comparison is possible only by constantly adjusting the tube containing potassium permanganate solution to the same point of the scale (10). This difficulty can be overcome by the use of the "absolute colorimeter" of Leitz, which permits colorimetric estimation against a special "grey solution" in monochromatic light, at the wave-length of the maximum absorption of the new test. Concerning the details of the colorimetric estimation we refer to Rosenthal and Erdélyi [1934, 1, 2]; here it may suffice to mention that this new method makes it possible to express the vitamin A content in "grey units."

This new method has been employed for direct determination of vitamin A in blood. At first we proceeded by extracting the serum or plasma with light petroleum. This extract, containing both carotene and vitamin A, was shaken five times in a separating funnel with 86 % methyl alcohol, after which treatment carotene had passed into the top layer (petroleum) whilst vitamin A was contained in the bottom layer (methyl alcohol). The collected and united alcoholic extracts were then diluted with an equal part of distilled water and extracted again with light petroleum, which this time removed the vitamin A. The petroleum extracts were washed with water, dried and evaporated at a low temperature *in vacuo*, and the residues were dissolved in absolute chloroform and used for determination of carotene and vitamin A, respectively.

Later the method has been simplified by the observation that the blue colour given by carotene with antimony trichloride disappears on warming with guaiacol; small amounts of carotene, such as occur in blood, do not therefore interfere with the direct determination of vitamin A.

The final method for determination of vitamin A is thus as follows. The carotene content of the blood to be taken should not exceed the threshold value mentioned (5γ in 1 ml. extract). 10–30 ml. of blood are extracted four times in a separating funnel with 50 ml. of light petroleum (B.P. $< 55°$). The greater the quantity and the lower the specific gravity of the petroleum, the more easily can emulsification be avoided. Should emulsification nevertheless occur, the fluid may be treated with a few ml. of petroleum and centrifuged at low speed for 3–4 minutes.

Addition of one drop of 96 % alcohol to the emulsion may also be of assistance. If alcohol has been employed, the petroleum extract must be thoroughly washed twice with distilled water. The collected petroleum extracts are dried over a minimum amount (0·01–0·02 g.) of anhydrous sodium sulphate for 2 hours, repeatedly filtered through dry filters and evaporated *in vacuo* at 40°. In order to remove the last traces of water the residue is dried once more for 20–30 minutes *in vacuo*.

The residue is tested as follows. Since such small quantities of vitamin may produce a very faint colour the final volume of the solution is reduced to 1 ml. (instead of 5 ml.) and the determination is made with the micro-cell of the Leitz absolute colorimeter. To the dry residue of the extract 0·25 ml. of absolute chloroform, 0·25 ml. of 5 % guaiacol in absolute chloroform and 0·5 ml. of a saturated solution of $SbCl_3$ are added. The mixture is put into a water-bath at 55° for 1½ minutes exactly. During this time the initial blue colour changes into purplish red. Both temperature and duration of warming should be adhered to exactly in order to keep volatilisation of chloroform within constant limits.

It would be most desirable to use a solution of pure vitamin A of known concentration as a standard. As we had no such preparation at our disposal, we had to express the quantity of vitamin A in another way.

A very simple method of comparison was given by the close similarity of the final colour of our reaction to that of 0·001 % potassium permanganate solution. Reading can take place in white or in monochromatic light corresponding to the maximum absorption of the colour in question. In this latter case we used light filter No. 7 (greenish yellow) of the Leitz absolute colorimeter.

The following data will show the results of several determinations (reduced to 1 ml. of serum):

Blood samples	Colorimeter readings with permanganate	The same values expressed in "grey units", calculated to 100 ml. of serum
1	2·71	5·4
2	3·84	7·6
3	2·85	5·5
4	4·05	8·3
5	3·12	6·2

In another method of colorimetric examination we used the "grey solution". In this case we fixed the cell containing 1 ml. of "grey solution" at 10 mm. and varied the layer of the solution to be tested. From the reading of the unknown and from the volume of the serum tested, the value of "grey units" corresponding to the vitamin A content of 1 ml. of serum may be calculated. The following equation serves for conversion into "grey units":

$$\text{Grey units} = \frac{\text{Reading of standard}}{\text{Reading of unknown} \times \text{ml. of serum}}.$$

If we could work with pure vitamin A, the intensity of the colour produced would be proportional to the absolute amount of vitamin A.

It cannot be foreseen what results of clinical and pathological importance may arise from the systematic examination of vitamin A in the blood, but, at any rate, it seems that the method described will furnish interesting and reliable data.

SUMMARY.

1. The new method of determining vitamin A in blood makes it unnecessary to remove carotene, since this substance does not react in the guaiacol test.

2. The new reaction thus permits direct determination of vitamin A in blood.

3. A dilute solution of potassium permanganate or the "grey solution" of the Leitz absolute colorimeter may be used as standards for comparison.

4. Until it becomes possible to determine the absolute amount of vitamin A with the aid of this new colour test, its expression in "grey units" seems suitable for research purposes.

REFERENCES.

Connor (1928). *J. Biol. Chem.* **77**, 619.
van Eekelen (1931). *Acta Néerl. Physiol.* (quoted by Menken).
Euler and Steffenburg (1928). *Z. physiol. Chem.* **175**, 38.
Menken (1932). *Deutsch. med. Woch.* 1484.
Rosenthal and Erdélyi (1934, 1). *Biochem. J.* **28**, 41.
—— —— (1934, 2). *Biochem. Z.* **271**, 414.
Rösiö (1929). *Z. physiol. Chem.* **182**, 289.
Wendt (1935). *Klin. Woch.* 9.

CXXX. THE RESPONSE OF THE ISOLATED UTERUS OF THE GUINEA-PIG TO HISTAMINE.

By HAROLD JOHN PHELPS.

From the Physiology Institute, Cardiff.

(*Received March 26th, 1935.*)

RESEARCHES carried out in the last few years have led to the view that true adsorption of weak electrolytes from aqueous solutions by neutral adsorbing surfaces proceeds through the medium of unionised molecules. Studies of adsorption by charcoal and fuller's earth have shown clearly the distinction between true adsorption and the apparent adsorption resulting from the interaction of dissolved ions with polar groups in the adsorbing surface [Fromageot and Wurmser, 1924, 1, 2; Phelps and Peters, 1929; Phelps, 1929; 1931, 1, 2].

It is obviously most pertinent from the biological standpoint to enquire whether the living cell takes material from its surroundings by true adsorption processes or by a mechanism of ionic exchange. It is of course very probable that the transfer of material from the fluid surrounding the cell to the fluid within the cell takes place by a migration of ions, and indeed the Donnan theory of membrane equilibria provides a theoretical basis for such a migration and, in general, gives a quantitative explanation of the observed facts. Such a scheme, however, takes no account of any specific concentration at the cell boundaries which may result from adsorption processes. Since many of the chemical reactions taking place in the living cell appear to be surface reactions it is reasonable to suppose that they must be preceded by adsorption processes.

This point can plainly be tested experimentally with certain pharmacologically active weak electrolytes, by comparing their pharmacological activities in acid and alkaline media. It is to be expected, for example, that a pharmacologically active base which ionises over the physiological range of p_H will, if it is taken up by the cell by a process of true adsorption, show greater pharmacological activity in alkaline media, in which, of course, the proportion of unionised molecules will be higher. From an acidic electrolyte the reverse result would be expected. Experiments on these lines have been reported from time to time. In particular may be mentioned the work of Andrus [1924] which demonstrated that adrenaline and tyramine had more powerful effects on the auricles of the rabbit heart at p_H 8 than at p_H 7. On the other hand choline and its esters which are presumably potential acids, were more actively depressant in acid media. Clark [1928] has reached a similar conclusion in the case of the action of acetylcholine on the frog's heart and has provided a technical explanation of the opposite result obtained by Voss [1926].

More recently Trevan and Boock [1927] have carried out a comprehensive study of the influence of p_H on the action of certain local anaesthetics on the eye. The correlation between anaesthetising action and the concentration of unionised molecules, as calculated from the known ionisation constants, is very striking. Labes [1922] has shown that atropine and cocaine are markedly less toxic in acid solutions and Werner and Blanchard [1929] have reported a similar result with strychnine. Zipf [1927] finds that frogs' livers take up alkaloids and basic dyes more readily from alkaline than from acid solutions. The same author

[1928] also finds that the contracture of frog's muscle produced by caffeine, quinine, nile blue *etc.*, is increased by increased alkalinity. Remarkably enough Zipf concludes from these results that the uptake of basic substances by tissues is a simple chemical replacement of free basic groups in the surface.

It is of course plain that any of these results considered individually is capable of two interpretations. Either it may be assumed that the neutral molecule of the stimulating substance is specifically active, or, on the other hand one must assume, as does Zipf, that increasing alkalinity induces some change in the cell surface itself which increases its responsiveness by increasing its adsorbing power or otherwise. When the results are considered collectively however the second interpretation seems most improbable. Trevan and Boock, for example, find that the anaesthetising actions of a number of substances in media of different reaction are in every case proportional to the concentration of unionised molecules as calculated from the known ionisation constants of the anaesthetics. If one may not assume that it is the unionised molecule which is adsorbed by the anaesthetised tissues, one must assume that the cell surfaces are so altered by change in reaction that their adsorbing power for each one of a number of different substances is also changed in such a way that in each case the change in adsorption happens to follow exactly the change in ionisation of the substance concerned, which surely is to assume an impossible degree of coincidence.

If however it is legitimate to argue from the analogous results obtained in the study of adsorption by charcoal and fuller's earth, it is surely reasonable to say that Zipf's results indicate rather a true "apolar" adsorption process.

In view of the strong presumptive evidence provided by these and similar investigations in favour of the view that the walls of the living cell take up substances from their surroundings by a mechanism of true "apolar" adsorption, it seemed desirable to study the influence of p_H on the pharmacological activity of some substance the adsorption of which by neutral adsorbents had already been studied. The influence of p_H on the adsorption of histamine has already been studied [Phelps and Peters, 1929]. From these results it appears that the adsorption of histamine increases rapidly over the range of reaction p_H 6–9. Since histamine is well known to have a marked pharmacological action on uterine smooth muscle it was thought that interesting results might be obtained by observing this action in media of varying reaction. It is true that Evans and Underhill [1923] have not found the action of histamine on the guinea-pig uterus to be influenced by changes in p_H, but as will appear in this paper, these workers did not employ a technique likely to disclose the small differences which might be expected to exist.

Work has been in progress on this problem for the last few years, and two brief reports have previously been published [Peters, 1929; Phelps, 1931, 3].

EXPERIMENTAL.

The guinea-pig uterus was chosen as providing suitable material, and the tests were carried out by a technique essentially similar to that described by Dale and Burn [1922] in their Medical Research Council Report on the Standardisation of Pituitary Extracts. The bath and fittings employed were of the modified design described by Gulland [1933].

The tests are beset with grave technical difficulties some of which may be mentioned. In the first place it was found (in confirmation of Dale and Burn [1922]) that not only do uteri from different animals show great differences in their response to histamine, but also that in any one preparation the relationship between response and dose is qualitative rather than quantitative and further that the sensitivity of the preparations generally varied considerably in the course of a series of tests. The Ringer solutions used have been made up to the composition recommended by Dale and Burn. Various methods of adjusting the reaction of the Ringer have been tried. It was found in the first instance that the maximum range of reaction which the guinea-pig uterus would tolerate without becoming subject to violent contractions in the absence of histamine was roughly p_H 7–8·5. Three obvious methods suggest themselves: (*a*) the use of definite buffer solutions such as phosphate or borate, (*b*) aeration of the testing bath with appropriate mixtures of oxygen and carbon dioxide, (*c*) adjustment of the p_H of the Ringer with dilute hydrochloric acid and reliance on the buffering effect of the bicarbonate in the solution to keep the reaction reasonably constant.

Although many successful experiments have been carried out using Ringer solutions buffered with phosphate mixtures to p_H 7·2 and p_H 8, this method has certain disadvantages. The Ringer solution as made up without phosphate has a reaction of about p_H 8·4, but the solution was buffered to a reaction somewhat less alkaline since there was a marked precipitation of calcium in more alkaline solutions in presence of phosphate. It has been established independently that diminution of the calcium content of the Ringer *per se* lowers the sensitivity of the uterus (Sir H. H. Dale tells me that this agrees with his own experience) so that the precipitation of calcium might mask in some degree the effect of change in reaction. The use of phosphate buffers was therefore abandoned.

It was not found possible to keep the isolated guinea-pig uterus alive for any considerable period in borate buffers.

Aeration of the testing bath with O_2/CO_2 mixtures was found to render the uterus preparation very unstable. This was very possibly due to the diffusion of free CO_2 gas into the tissues. On the other hand preadjustment of the bicarbonate balance by the addition of the requisite quantity of hydrochloric acid was found very satisfactory. A similar result could be produced by bubbling CO_2 into the Ringer before use, but more accurately reproducible results could naturally be obtained by adding measured quantities of acid. The actual procedure which was found most satisfactory was to make up a "stock" Ringer solution to the formula given by Dale and Burn [1922], calcium and magnesium chlorides being omitted (a trace of phenol red being added to facilitate subsequent adjustment of p_H). For use the necessary amounts of strong solutions of these salts were added. To the acid Ringer sufficient *N*/10 HCl was added to bring the reaction to approximately p_H 7·15. To the alkaline Ringer sufficient NaCl was added to make $[Cl^-]$ the same as in the acid Ringer.

The acid Ringer so prepared became more alkaline after aeration owing to loss of CO_2 but it was generally found possible to make histamine tests before the reaction had changed significantly, as indicated by the phenol red. It is assumed that, as an average, the more acid histamine tests were carried out at p_H 7·2.

By the use of the two-way tap at the bottom of the testing bath, the latter could be filled with the appropriately buffered solution. When the preparation had become stable a suitable dose of histamine was added as histamine acid phosphate in normal saline in a concentration of 1/50 mg. per ml. When the bath was normally filled the volume of the testing solution was about 80 ml.

The preparation was first sensitised by a dose of histamine more than enough to produce maximum contraction (usually 0·02–0·04 mg.). The difference in response to be expected between p_H 7·2 and p_H 8 is small, the amount of unionised histamine increasing over this range in the ratio of 1/1·3 approximately. The first step therefore was to discover the minimum dose of histamine that would produce a response in alkaline solution. In general this was 0·005–0·01 mg. in 80 ml. Minimum doses were then administered alternately in acid and alkaline media. As the experiment proceeded the sensitivity of the preparation usually altered somewhat and corresponding changes had to be made in the dosage. It was not to be expected that such small differences in response could be demonstrated in every experiment with such a tissue as the excised uterus, or indeed with any biological preparation. In the course of about thirty experiments however no single preparation has consistently given a greater response in acid than in alkaline solutions[1]. In many experiments no difference in response could be detected with certainty; in the majority however there was a definitely greater response to histamine in alkaline media. Tables I and II are protocols of two typical experiments which seemed free from technical fault; one with phosphate buffering and the other in which the reaction was adjusted with hydrochloric acid and in which the minimum dose fortunately was adjusted very exactly.

Table I.

Phosphate buffering; 369 g. guinea-pig.

Test	Histamine vol. solution 1/50 mg. per ml. ml.	p_H	Result
A	0·2	7·8	Large contraction
B	0·2	7·2	No contraction
C	0·2	7·8	Fair response
D	0·4	7·8	Good contracture
E	0·4	7·2	Practically equal to "D"
F	0·4	7·8	Equal to "D" and "E"
	(doses probably too large)		
G	0·3	7·8	Good contracture well maintained
H	0·3	7·2	Some contraction not maintained
J	0·3	7·8	Good contracture, maintained
K	0·1	7·2	Very slight contraction not maintained
L	0·1	7·8	Contracture much greater than "K"
M	0·1	7·2	Smaller contracture less maintained

Table II.

Bicarbonate buffering; 279 g. guinea-pig.

Test	Histamine vol. solution 1/50 mg. per ml. ml.	p_H	Result
A	0·5	8·4	Large contraction
B	0·5	7·2	No response
C	0·5	8·4	Slight contraction
D	1·0	7·2	Fair response, delayed
E	1·0	8·4	Large response
F	1·0	7·2	Slight irregular contraction
G	0·5	8·4	Large contracture
H	0·5	7·8	No response
J	0·5	8·4	Large response delayed
K	0·25	7·2	No response
L	0·25	8·4	Slight response
M	0·25	7·2	No response

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[1] It is of interest that the most stable preparations have been obtained in the summer months.

DISCUSSION.

It may reasonably be concluded from the results that the guinea-pig uterus shows an increased response to histamine in alkaline solutions, which might be expected if the pharmacological activity of histamine were due to the apolar adsorption of unionised molecules by cell surfaces.

The results of Evans and Underhill who failed to find any difference in the response and those of Hemingway [1926] who finds that the general responsiveness of living tissues is enhanced by alkali *per se* must be considered in the light of the present work. Since it has been observed that the difference in response is so small over the range of reaction in which the tissue may be regarded as in any way normal and can, in fact, only be demonstrated by administering minimum doses, it seems possible that the effect might have escaped the notice of Evans and Underhill entirely owing to the administration of doses in excess of the minimum dose.

Hemingway found that in general, uterus, the plain muscle of blood vessels and intestine in strongly alkaline solutions were pharmacologically hypersensitive. The effect persisted for some time in neutral media and probably depended on an alteration of the internal reaction of the cells, brought about by the large excess of alkali. These conditions are hardly comparable with those of this investigation. Further it was invariably the case that the preparations which I used were less stable and more responsive to purely mechanical stimulation at p_H 7 than at p_H 8, which adds support to the assumption that any increase in response found in alkaline solution must be a property of the stimulating agent rather than of the responding tissue.

It seems therefore that the guinea-pig uterus provides a further example of a tissue which responds to pharmacological reagents in the unionised form and of a tissue of which the cells take up at least some substances by a process of simple apolar adsorption.

In conclusion the author expresses his grateful thanks to Prof. R. A. Peters and Dr A. Hemingway for much valuable advice and to Dr H. M. Carleton for the loan of certain apparatus.

REFERENCES.

Andrus (1924). *J. Physiol.* **59**, 361.
Clark (1928). *J. Physiol.* **64**, 123.
Dale and Burn (1922). *Med. Res. Coun. Sp. Rep. Series*, No. 69.
Evans and Underhill (1923). *J. Physiol.* **58**, 1.
Fromageot and Wurmser (1924, 1). *Compt. Rend. Acad. Sci.* **179**, 972.
—— —— (1924, 2). *Compt. Rend. Acad. Sci.* **179**, 1404.
Gulland (1933). *Biochem. J.* **27**, 1216.
Hemingway (1926). *J. Physiol.* **62**, 81.
Labes (1922). *Biochem. Z.* **130**, 14.
Peters (1929). *J. State Med.* **37**, 1.
Phelps (1929). *J. Chem. Soc.* 1724.
—— (1931, 1). *Proc. Roy. Soc. Lond.* A **133**, 155.
—— (1931, 2). *Proc. Roy. Soc. Lond.* A **131**, 18.
—— (1931, 3). *Chem. Ind.* **50**, 1043.
—— and Peters (1929). *Proc. Roy. Soc. Lond.* A **124**, 584.
Trevan and Boock (1927). *Brit. J. Exp. Path.* **8**, 302.
Voss (1926). *Arch. exp. Path. Pharm.* **116**, 367.
Werner and Blanchard (1929). *Amer. J. Physiol.* **90**, 83.
Zipf (1927). *Arch. exp. Path. Pharm.* **124**, 259.
—— (1928). *Arch. exp. Path. Pharm.* **140**, 56.

CXXXI. CRYSTALLINE INSULIN.

By DAVID ALYMER SCOTT and ALBERT MADDEN FISHER.

Connaught Laboratories, University of Toronto.

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In a previous communication from these Laboratories [Scott, 1934] attention was directed to the importance of certain metals in the crystallisation of insulin. It was shown that zinc was a constituent of insulin crystals prepared by the pyridine-brucine method of Abel, or by the saponin method of Harington and Scott, and it was found that good yields of crystals were obtained when the chlorides of zinc, nickel, cobalt or cadmium were added to the insulin solution. In fact, it was evident that in cases where the amorphous insulin was free from ash, the addition of one of these metals was essential for the formation of crystals. Moreover, it was suggested that the success of the previously published methods for crystallising insulin depended in part upon the fact that zinc is present in pancreas, in commercial insulin preparations and as an impurity in saponin. We have recently been engaged in the determination of the ash content of samples of insulin crystallised from ammonium acetate buffers to which one of the above metals had been added. The results of this work, together with a discussion of some observations already recorded by one of us (D. A. S.), are presented in the present communication.

In the method of crystallisation previously reported it was shown that insulin crystals can be readily obtained from amorphous preparations of insulin dissolved in phosphate, sodium acetate or ammonium acetate buffers if traces of certain metals are present. There are however a number of minor differences between these buffers which should be borne in mind. In the first place it was found that the addition of about 10 % of acetone to the phosphate or sodium acetate buffers facilitated crystallisation. With ammonium acetate however the best yields of crystals were obtained when the buffer was free from acetone. In fact the addition of this reagent caused the formation of an amorphous, rather than a crystalline, precipitate. Secondly, insulin crystallises best from a phosphate buffer solution at acidities between p_H 6·0 and 6·2 whereas, using ammonium acetate buffer, crystallisation proceeds most readily between p_H 5·8 and 6·0. Thirdly, a phosphate buffer is the most convenient one to use when the work is such that it extends over a lengthy period, in that quantities of this solution can be stored for several months without any change in p_H. Ammonium acetate buffers involve the neutralisation of acetic acid solutions of insulin with ammonium hydroxide; since the strength of the latter solution does not remain constant, considerable manipulation is necessary to reach the requisite acidity of the neutralised insulin solution. Fourthly, insulin crystals prepared by the phosphate buffer method are generally sharper in outline than those separated from an ammonium acetate solution. On the other hand ammonium acetate buffers have a decided advantage over phosphate buffers in the crystallisation of insulin by means of heavy metals in that the former yield crystals of lower ash content; this may perhaps be due to the greater solubility of the acetates of heavy metals as compared with the phosphates. Since it is desirable to have crystalline insulin with as low an ash content as possible, it is therefore advisable to subject the material to a final recrystallisation from ammonium acetate. We have found it convenient to conduct the initial crystallisation of insulin in a phosphate buffer solution containing zinc chloride and acetone, and to recrystallise this material from ammonium acetate without acetone or the further addition of metals. Insulin crystals prepared in this manner have an ash content of 0·65 % when zinc is the metal used to aid in the first crystallisation.

It was previously shown that a large part of the ash could be removed from insulin crystals by precipitating the insulin from aqueous 0·1*N* HCl with the aid of alcohol and ether. Dissolving and reprecipitating the insulin as the hydrochloride a second time however did not materially decrease the ash content. Since zinc chloride is reasonably soluble in acid alcohol, and since a large proportion of the zinc is removed from crystalline insulin by this solvent, it is difficult to understand why an ash-free product is not obtained by this treatment. We recently carried out a great number of experiments to clarify this problem. In this work we dissolved insulin containing 0·7 % ash in various concentrations of hydrochloric acid and precipitated it as the hydrochloride with alcohol and ether, acetone, methylethylketone or dioxan. The ash content of the resultant product was usually 0·2 %. On one lot of crystals an ash content of 0·05 % was regularly obtained when acetone was used. We are at present unable to offer a satisfactory explanation for this phenomenon. It may be that in certain commercial preparations of insulin there is some other metal the chloride of which is not soluble in high concentrations of alcohol and ether, or in acetone. This metal may or may not aid in the formation of insulin crystals. That it is not essential for crystallisation however is shown by the fact that an insulin hydrochloride having an ash content of only 0·02 % crystallises readily in the presence of traces of zinc, nickel, cobalt or cadmium.

In the experiments recorded in the previous paper it was noted that after electrodialysis and precipitation of the insulin as the hydrochloride by alcohol and ether, there was a slight loss in insulin activity. Recent experiments have shown that the loss in activity did not occur during electrodialysis but did occur during the preparation of the insulin hydrochloride. Carr *et al.* [1929] were the first to observe that acid solutions of insulin were inactivated by alcohol and suggested that the inactivation was due to esterification. In our experiments on the precipitation of insulin as the hydrochloride by means of alcohol and ether, it seemed scarcely possible that inactivation was due to esterification since the precipitation of the insulin was effected almost immediately after solution. Nevertheless, to eliminate the possibility of such a reaction, all our recent experiments have been conducted by precipitating the insulin as the hydrochloride by means of acetone or methylethylketone. The acetone was first purified by boiling for 20 hours with potassium permanganate and was then distilled. From a large number of experiments on the precipitation of insulin as the hydrochloride with acetone, varying results have been obtained. In about 60 % of the experiments there was a loss in potency of approximately 15 % during precipitation. In two experiments a 20 % increase in activity was obtained. This material however after being kept in a dry state for 2 months, lost 50 % of its activity. In the remaining experiments no change in activity was detectable. When inactivation occurred, attempts were made to reactivate the insulin with alkali. Some measure of success was attained but the original activity of the crystalline insulin was never secured. These facts again raise the question of the inactivation of insulin with various solvents. We do not believe that it is due to the reducing action of traces of metals since the same insulin can be kept in an aqueous acid solution for several weeks without any loss in activity, nor are we inclined to believe that it is due to esterification since no evidence of such has been found by analytical procedures [Charles and Scott, 1931]. Moreover, inactivation takes place with such solvents as acetone and methylethylketone where esterification is impossible. This inactivation may be due to some molecular rearrangement such as a tautomeric change or to the opening or closing of a ring structure. The proof of such theories however must wait until more is known of the chemical structure of insulin.

The observation was made in the former publication that the insulin after electrodialysis did not flocculate until p_H 6·3 was reached when neutralised with sodium hydroxide. We have verified this observation many times during the past few months and have found that the acidity at which an electrodialysed ash-free product flocculates is between p_H 6·1 and 6·3. Wintersteiner and Abramson [1932–33] found that insulin crystals suspended in *M*/30 sodium acetate buffers are isoelectric at p_H 5·0 and have an isoelectric point of p_H 5·3 as determined by electric mobility experiments using insulin absorbed on quartz in *M*/30 acetate. The difference between the isoelectric point of insulin as found by these authors and the point of maximum precipitation as observed in our experiments may be due to a different concentration of electrolytes. However, another view might be considered. It is possible that after neutralising the dialysed insulin with sodium hydroxide or ammonium hydroxide the amorphous sodium or ammonium salt of insulin is formed. We have repeatedly found that the best acidity for the crystallisation of insulin in the presence of certain metals is between p_H 6·0 and 6·2. If crystallisation is due to the combination of these metals with the free carboxyl groups of insulin, then this process should take place more readily on the alkaline side of the isoelectric point. In other words, crystalline compounds of insulin with various metals should have the characteristics of a substance having an isoelectric point more alkaline than that of the ash-free material. Similarly, if the sodium or ammonium salt of insulin is formed on neutralising a solution of insulin after dialysis, the point of maximum precipitation would be on the alkaline side of the isoelectric point of insulin.

During the past few months a great amount of experimental work has been done on insulin hydrochloride of low ash content. It was felt that should crystalline insulin be a complex compound of two or three chemical entities (such as proteins) bound to each other by combination with a metal, then, after the removal of the metals, there might be a possibility of obtaining a more active insulin by fractional precipitation. Using insulin with an ash content of 0·05 % we have tried fractionation experiments with alcohol and with acetone. This work has been carried out over a wide range of acidities, using either sulphuric acid or hydrochloric acid. Fractionation was also attempted at the isoelectric point with these solvents. In some cases, the solvents were added to insulin solutions which had been made alkaline with sodium hydroxide or with ammonium hydroxide. None of these experiments resulted in a successful fractionation of the insulin.

We have also determined the ash content of insulin crystals formed by the recrystallisation of an ash-free insulin. The metals used were zinc, cadmium and cobalt. The experimental work is presented in detail below.

EXPERIMENTAL.

For the purposes of this research, a large quantity of crystalline insulin was prepared. The first crystallisation was conducted in a phosphate buffer solution containing zinc chloride. The crystals were removed, washed and dried and then recrystallised from ammonium acetate at room temperature without acetone or the further addition of metal. The final product had an ash content of 0·7 % and contained 25 I.U. of insulin per mg.

From this material an ash-free insulin was obtained by the following treatment. 0·5 g. of the crystals was placed in each of two 250 ml. centrifuge-tubes. To each tube were added 10 ml. of 0·1 N HCl. As soon as the crystals had dissolved, 200 ml. of acetone were added to each. The insulin readily precipitated. The tubes were placed in a refrigerator for 2 hours. The mixtures were then centrifuged, the acetone decanted and the precipitates combined by immediately dissolving them in 500 ml. of water containing 2 ml. of N HCl. The solution was then transferred to a litre beaker and electrodialysed using the type of apparatus formerly described. The solution in the beaker was vigorously stirred with a mechanical stirrer. Dialysis was continued for 1¼ hours, during which time 35 litres of acid water, p_H 2·3, circulated through the thimbles. After the completion of dialysis, the solution was filtered into a 600 ml. beaker and very slowly neutralised with N NH_4OH until maximum precipitation was reached. The mixture was placed in the refrigerator overnight. The supernatant liquid was then decanted and the precipitate centrifuged in a 250 ml. tube. The clear solutions were combined and the p_H was determined and was found to be 6·2. The precipitated insulin was then dissolved in 15 ml. of 0·1 N HCl. After the addition of 100 ml. of acetone, the solution was divided into two equal parts and the insulin was precipitated by adding 150 ml. of acetone to each sample. The mixtures were then placed in the refrigerator overnight. After centrifuging, the supernatant liquids were discarded and the precipitates dried *in vacuo*. Samples of the dried insulin hydrochloride were then removed for ash estimations which gave the values of 0·01 and 0·03 %. The test for zinc was faintly positive. A physiological assay in which 300 mice were used showed that the material contained 23 I.U. per mg. From this material the various crystalline metallic salts were prepared and their respective ash contents were determined.

Samples of this ash-free insulin hydrochloride were crystallised by means of various metals from ammonium acetate. Ammonium acetate was chosen in preference to phosphate buffer for reasons already stated. Acetic acid, which yielded no residue on evaporation, and freshly distilled ammonium hydroxide were used to prepare $N/3$ solutions. 100 mg. of the insulin powder were weighed into a dry beaker and 50 ml. of $N/3$ acetic acid were added. After the insulin had dissolved, 5 ml. samples of the solution were measured into each of ten 15 ml. centrifuge-tubes previously treated with cleaning solution and washed several times with distilled water. 0·1 ml. of 0·5 % $ZnCl_2$ was added to each sample. The acidity was adjusted to approximately p_H 5·9 with $N/3$ NH_4OH. The tubes were inverted to ensure thorough mixing (but were not scratched) and kept at room temperature for 2 hours. They were then put in the refrigerator overnight. At the same time a control tube was prepared containing 0·5 ml. of the zinc chloride solution in the same volume of buffer, but no insulin. The following morning each tube except the control contained well-defined crystals. About 5 ml. of each supernatant liquid were decanted and the precipitates from every two tubes mixed, centrifuged and the supernatant liquids discarded. The five crystalline precipitates thus obtained were allowed to drain for 15 minutes by inverting the tubes over filter-paper. 2 ml. of distilled water were then measured into each tube and the crystals washed by gentle shaking. After centrifuging, the tubes were allowed to drain for 15 minutes over filter-paper. This washing process was repeated three times; during this washing, any amorphous material was carefully removed from the crystals. After the final washing and draining, the precipitates in each of the five tubes were dried *in vacuo*, and the ash content of each sample was determined. These values are reported in Table I.

Table I. *Ash estimations of zinc-insulinate.*

Weight of crystals mg.	Weight of ash (ZnO) mg.	ZnO %		Zn %
8·09	0·048	0·60		0·48
8·92	0·060	0·67		0·54
12·31	0·089	0·72		0·58
7·71	0·050	0·65		0·52
7·84	0·043	0·55		0·44
10·61	0·070	0·70		0·56
			Mean	0·52

The insulin crystals which were formed with cadmium and cobalt were prepared in a similar manner except that 0·1 ml. of 0·5 % $CdCl_2$ or 0·1 ml. of 0·5 % $CoCl_3$ was added to each tube instead of the zinc chloride. The crystals were washed as previously described. The ash contents are shown in Tables II and III.

Table II. *Ash estimations of cadmium-insulinate.*

Weight of crystals mg.	Weight of ash ($CdSO_4$) mg.	$CdSO_4$ %		Cd %
8·14	0·118	1·45		0·78
8·49	0·113	1·33		0·72
6·88	0·104	1·51		0·81
			Mean	0·77

Table III. *Ash estimations of cobaltous-insulinate.*

Weight of crystals mg.	Weight of ash (Co_3O_4) mg.	Co_3O_4 %		Co %
5·40	0·033	0·61		0·45
7·91	0·049	0·62		0·46
8·01	0·044	0·55		0·41
			Mean	0·44

Further quantities of crystalline insulin were prepared in the manner described above, using zinc chloride. After washing and drying, a sample of the material was removed and found to contain 0·7 % ash. The remainder was recrystallised from an ammonium acetate buffer without further addition of metal. The ash of this material was also 0·7 %.

Ash determinations were made by burning a known weight of the crystals in a current of oxygen [Emich-Schneider, 1932]. Difficulty was encountered in determining cadmium as the oxide because of the volatility of this substance. Hence it was estimated as the sulphate by adding one or two drops of strong sulphuric acid to the sample before ashing. The ash from the zinc experiments gave a negative chloride test.

We have also determined the nitrogen contents of a number of samples of insulin crystallised from phosphate and from ammonium acetate buffers using zinc chloride. A series of eight samples of crystals was prepared from sodium phosphate and a similar series from ammonium acetate. In the former series, the buffer was neutralised with $N/3$ NaOH instead of NH_4OH. After centrifuging, the crystals were washed and dried as already described. The average of eight estimations on each series showed that the crystals isolated from the phosphate buffer contained 14·0 % N while those prepared from the ammonium acetate buffer contained 14·7 % N.

DISCUSSION.

From a quantity of insulin hydrochloride having an ash content of 0·02 % and containing 23 I.U. per mg., several crystalline preparations of insulin were made. Crystallisation of the insulin was conducted in an ammonium acetate buffer solution containing traces of either zinc, cobalt or cadmium. All the crystals had the same microscopic appearance. The ash contents of the various kinds of crystals are reported in Tables I, II and III, from which it will be observed that the agreement between the ash values of different preparations of the same insulin salt is within the limits of experimental error and further that the percentage of ash varies with the metal used to aid in the crystallisation of the insulin.

The problem of the formula weight of insulin has been discussed by at least two groups of workers. Basing their estimate on data obtained by chemical treatment, Freudenberg and Dirscherl [1931] have advanced the suggestion that the formula weight of insulin may be 10,000 or 20,000. Accepting the latter figure, and using the data of Tables I, II and III in the present paper, a simple calculation shows that each formula weight of insulin contains 1·6 formula weights of zinc, 1·4 formula weights of cadmium or 1·5 formula weights of cobalt. If however we assume double the value suggested by these authors, then the metallic content of the crystals, in formula weights, can be expressed as whole numbers within the limits of experimental error. Using the ultracentrifuge, Svedberg [1931] estimated the formula weight of insulin to be 35,100. However, our knowledge of the chemistry of insulin may still be advanced without having an exact value for its formula weight.

Crystalline preparations of insulin containing either zinc, cadmium or cobalt have been described in this paper. It was found that the ash content of each sample was proportional to the formula weight of the metal which it contained. Furthermore, this ash value seemed to be characteristic in that it was not decreased by recrystallisation. These facts suggest that the metal occurs as a chemical constituent of the crystals and not merely as an impurity. The manner in which each metal is combined cannot however be decided. One might advance the suggestion that a metallic salt had been formed through the carboxyl groups. The large proportions of the dibasic amino-acids, glutamic acid and cystine in this hormone [Jensen and Wintersteiner, 1932] seem to bring this idea within the realm of possibility. If the formula weight of insulin is 40,000, the metallic content of the crystals could account for six carboxyl groups. This represents but a small number of the possible free carboxyl groups from the dibasic acids in insulin. On the other hand, the formation of protein salts through free carboxyl groups has been questioned by Robertson [1924]. The tendency of zinc, cadmium and cobalt to form many complex salts with amines should not be overlooked. There also remains the possibility of formation of a ring structure by combination of these metals with both amino- and carboxyl-groups.

In our work on the crystallisation of insulin it was found that crystals prepared from an ammonium acetate buffer had a higher nitrogen content than those obtained from a sodium phosphate buffer. In the past this fact has been attributed to traces of ammonium acetate adhering to the crystals. However, since repeated washing of the crystals failed to decrease the nitrogen content, we are inclined to believe that the nitrogen is chemically combined. Here again, combination through carboxyl groups could be suggested. It is also reasonable to suppose that the ammonia has formed a molecular complex with the metal in the insulin, for it is well known that certain salts of cobalt form as many as six molecular complexes with ammonia.

SUMMARY.

A quantity of insulin hydrochloride having an ash content of only 0·02 % was prepared. Different samples of this insulin were crystallised from an ammonium acetate buffer solution by means of zinc, cobalt or cadmium. Ash estimations made on different samples of the crystalline products showed constant values for the respective metals. Further, the average ash content of each insulin salt was proportional to the atomic weight of the metal which it contained. This fact strongly indicates that crystalline insulin contains the metals as chemically combined constituents and not as impurities. The percentage ash values are discussed in respect to certain observations regarding the chemistry of insulin. The crystallisation of insulin and certain other aspects of earlier work on this problem are also considered.

REFERENCES.

Carr, Culhane, Keeller and Underhill (1929). *Biochem. J.* **23**, 1010.
Charles and Scott (1931). *J. Biol. Chem.* **92**, 289.
Emich-Schneider (1932). Microchemical laboratory manual. (London.)
Freudenberg and Dirscherl (1931). *Z. physiol. Chem.* **202**, 192.
Jensen and Wintersteiner (1932). *J. Biol. Chem.* **98**, 285.
Robertson (1924). The physical chemistry of the proteins. (London.)
Scott (1934). *Biochem. J.* **28**, 1592.
Svedberg (1931). *Nature*, **127**, 438.
Wintersteiner and Abramson (1932–33). *J. Biol. Chem.* **99**, 741.

CXXXII. ZINC CONTENT OF BOVINE PANCREAS.

By ALBERT MADDEN FISHER and DAVID ALYMER SCOTT.

Connaught Laboratories, University of Toronto.

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In a recent publication from these Laboratories, data were presented concerning the relative quantities of insulin which could be extracted from the pancreas of cows and calves of various age groups [Fisher and Scott, 1934]. It was found that the yield of insulin per kg. of young foetal pancreas was about twenty times greater than that of the pancreas of old cows. It has also been shown that certain metals have a very close association with insulin in that traces of zinc, nickel, cobalt or cadmium facilitated the crystallisation of this hormone [Scott, 1934]. Further, commercial preparations of insulin and also insulin crystals prepared by the brucine or saponin methods gave positive tests for zinc. These facts suggested that certain of the metals might be associated with insulin in the pancreas. If such were the case, it would be of interest to determine the quantity of each of these metals in bovine pancreas of various age groups and to correlate the values, if possible, with the amount of insulin found in the pancreas of similar age groups. An investigation of this problem is reported in the present paper.

Many workers have reported the occurrence of zinc in the pancreas of various species [Lutz, 1926; Delezenne, 1919]. The presence of cobalt and nickel is also the subject of other communications [Bertrand and Macheboeuf, 1926]. None of these researches however was performed with reference to the age of the animals from which the pancreas was obtained.

In the present work it was decided to determine the relative quantities of cobalt and nickel in various samples of pancreas by means of some of the well-known colour tests for these metals. Zinc could be determined either by the fluorescence method of Lutz or gravimetrically in the manner outlined by Delezenne. In view of the difficulty of securing a sufficient quantity of satisfactory stercobilin used in the former method, it was decided to follow the procedure described by Delezenne. Moreover, this method obviates the necessity of dry ashing—a feature which has often been criticised on the ground of the possible loss of zinc by volatilisation.

EXPERIMENTAL.

The pancreatic glands were removed from cattle of the following age groups: hairless foetal calves, under 5 months; haired foetal calves, 5–7 months; calves, 6–8 weeks; and cows, 9 years and over. In the older age groups, the glands from at least 10 animals were minced and an aliquot was removed for the investigation. For the foetal groups, it was necessary to collect the glands from a much larger number in order to obtain samples of sufficient size for the determinations. The glands in each group were minced, weighed and kept *in vacuo* over concentrated sulphuric acid for several days. After the tissue had been thoroughly dried, the glands were weighed again. For the determinations, 15 or 20 g. samples of this material were placed in 500 ml. Erlenmeyer flasks.

The procedure used in the estimation of the metallic content of each of these weighed samples was as follows. A solution of equal parts of concentrated sulphuric and nitric acids was added dropwise to the tissue, using about 1 ml. for each g. of tissue. The flask was shaken and very gently heated until copious fumes were formed. After cessation of fuming, the heating was resumed and concentrated nitric acid added dropwise. Vigorous heating and the addition of nitric acid were continued until a pale yellow solution was obtained. This was poured into a small porcelain evaporating dish and evaporated to dryness. Washings from the original flask were added from time to time. The dry white residue was intimately mixed with about 10 ml. of concentrated hydrochloric acid and then evaporated to dryness. The residue was again stirred with 10–15 ml. of concentrated hydrochloric acid and washed into a 15 ml. centrifuge tube. After centrifuging the supernatant liquid was measured and poured into a 50 ml. beaker. The spot tests described by Feigl [1931] for cobalt and nickel were conducted on this solution. For the former metal we used the various modifications of tests with α-nitroso-β-naphthol. Dimethylglyoxime tests described by Feigl were used for nickel. With all samples of pancreas used in this investigation, tests for cobalt and nickel were negative. As a control, drops of the various reagents were added to one drop of a solution containing 0·015 mg. of nickel per ml. or 0·030 mg. of cobalt per ml. One drop of such a standard solution contained sufficient nickel or cobalt to give a strongly positive test, either when the test was conducted directly upon the standard solution acidified with one drop of concentrated hydrochloric acid or when one drop of the standard was mixed with one drop of the unknown solution.

Upon completion of the spot tests for cobalt and nickel, the procedure for the determination of zinc was resumed. The unknown solution in the beaker was gently boiled until the volume had been decreased to about 5 ml. After adding an equal volume of water, the acid solution was poured slowly into 40 % of its volume of concentrated ammonium hydroxide—sufficient to render the mixture distinctly ammoniacal. About 3 ml. of a suspension of calcium hydroxide and a few drops of "superoxol" were added. After standing for a few hours, the mixture was centrifuged and the supernatant liquid decanted into a 200 ml. flask. The precipitate was dissolved in concentrated hydrochloric acid and reprecipitated with ammonium hydroxide. After centrifuging, the supernatant liquid was added to that from the first precipitation. This solution was boiled and a suspension of calcium hydroxide was added from time to time, until the evolution of ammonia could not be detected by moist litmus paper, even when the solution itself was distinctly alkaline. The mixture was centrifuged in a 50 ml. pointed centrifuge-tube and the precipitate washed with distilled water. The precipitate was then dissolved in hydrochloric acid, using a sufficient quantity to ensure that the final solution contained 5–10 % hydrochloric acid. The solution was saturated with hydrogen sulphide, the tube stoppered and kept overnight. After centrifuging, the supernatant liquid was transferred to a small porcelain dish and evaporated to dryness. The residue was stirred thoroughly with four 10 ml. quantities of a 10 % solution of ammonium acetate acidified with glacial acetic acid. The clear solution was then transferred to a 50 ml. pointed centrifuge-tube and the zinc precipitated by saturation with hydrogen sulphide. The tube was stoppered and kept for 3–4 days. The mixture was then centrifuged and the white precipitate of zinc sulphide dissolved in a few ml. of concentrated sulphuric acid. This solution was slowly evaporated to dryness on a weighed platinum dish. Strong heating was avoided in order to eliminate the possible decomposition of the zinc sulphate noted by Delezenne.

From the weight of the residue—zinc sulphate—the amount of zinc per kg. of pancreas was calculated. Similar determinations were carried out for each age group. The values are recorded in Table I.

Table I. *Zinc content of bovine pancreas.*

Description of animal	Pancreas		Zinc per kg. dry tissue mg.	Zinc per kg. wet tissue mg.	Insulin per kg. wet tissue* I.U.
	Wet weight g.	Dry weight g.			
Cows	500	130	57	15	1,800
(9 years and over)			80	21	
			60	16	
Calves	200	56	90	25	11,400
(6–8 weeks)			75	21	
Foetal calves	370	94	55	14	23,000
(haired, 5–7 months)			70	18	
			75	19	
Foetal calves	155	33	153	33	33,200
(hairless, under 5 months)					

* Fisher and Scott [1934].

DISCUSSION.

The zinc content of bovine pancreas, reported in Table I, shows no relation between the age of the cattle and the metallic content of the glands. The pancreas was found to contain about 20 mg. of zinc per kg. of fresh tissue. This value is in agreement with that reported by Delezenne. The fact that there is no simple relation between the zinc content and the amount of insulin which can be extracted from the glands is not surprising when it is remembered that crystalline zinc insulinate contains only 0·5 % zinc. There is thus in the pancreas many times the quantity of zinc necessary to account for the available insulin in the pancreas as the zinc salt.

The fact that the presence of nickel and cobalt was not detected in the pancreas of cattle of any age group may mean that only traces of these metals are present and that the amount is beyond the sensitivity of our method. Bertrand and Macheboeuf [1926] reported 0·715 mg. of nickel and 0·357 mg. of cobalt per kg. of dried ox pancreas. Although there is a wide difference between some of the values reported by them for ox pancreas, there is no significant difference between the quantity of these metals in ox pancreas and that found in calves' pancreas. Their values were obtained by comparing the weight or colour of the compounds which these metals form with dimethylglyoxime in their unknown solutions with the weight or colour formed by a known quantity of nickel or cobalt with the same reagent. They used samples of approximately 200 g. In our work it would be rather difficult to obtain sufficient foetal pancreas to conduct duplicate determinations with this size of sample. In view of the fact that, in the present investigation, tests for these metals were positive when a drop of the standard nickel or cobalt solution was added to the unknown solution, our inability to detect these metals in the pancreas was not due to the presence of an interfering substance.

SUMMARY.

Bovine pancreas was found to contain about 20 mg. of zinc per kg. of fresh tissue, irrespective of the age of the animal from which the gland was obtained. The quantity of insulin which can be extracted from bovine pancreas bears no simple relation to the amount of zinc contained in the glands. Cobalt and nickel were not detected in the pancreas.

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REFERENCES.

Bertrand and Machebocuf (1926). *Compt. Rend. Acad. Sci.* **182**, 1305.
Delezenne (1919). *Ann. Inst. Pasteur*, **33**, 78.
Feigl (1931). Qualitative Analyse mit Hilfe von Tupfelreaktionen. (Leipzig.)
Fisher and Scott (1934). *J. Biol. Chem.* **106**, 305.
Lutz (1926). *J. Ind. Hygiene*, **8**, 177.
Scott (1934). *Biochem. J.* **28**, 1592.

CXXXIII. THE EFFECT OF HALOGEN SALTS ON THE CLOTTING OF MILK BY TRYPSIN.

By WINIFRED MARY CLIFFORD.

From the Physiology Department, King's College of Household and Social Science, Campden Hill Road, W. 8.

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Whilst working on the effect of halogen salts on steaptic digestion [Clifford, 1934], it was noticed that many of them enabled trypsin to coagulate milk. Since such a function is generally ascribed to Ca alone, it was decided to carry out further experiments.

Mellanby [1912] investigated the coagulation of milk by trypsin but added $CaCl_2$ in nearly all his experiments; he also compared the action of the latter with those of $MgCl_2$ and $BaCl_2$, finding that they also increased the milk clotting activity, though in a less degree than did the Ca salt.

In the same year Mellanby and Woolley [1912] stated that enzymes acting in an alkaline medium, *e.g.* trypsin, need extra Ca to produce typical coagula in milk.

This idea was followed up by Seth [1924] who used $CaCl_2$-milk to investigate tryptic activity, and still later Kleiner and Tauber [1934, 1, 2] found the clotting of $CaCl_2$-milk to be a function of low concentrations of crystalline trypsin. They did not try the effect of adding any other salt.

Experiments were therefore made to determine the effect of various halogen salts on tryptic clotting.

Experimental.

The method used was that described in a previous paper [Clifford, 1927] on the effects of halogen salts on peptic milk clotting.

10 ml. of milk and 2 ml. of water, or halogen salt solution of the requisite concentration, were warmed to 37°. To this mixture was added 1 ml. of a 2 % solution of pancreas substance. The tube was inverted to mix, and the time of clotting was determined by means of a stop-watch.

An average of the times of clotting of six tubes was taken for each concentration of salt used, and in no set of six was the difference greater than 10 seconds.

Three separate batches of commercial pancreas substance (2 powdered and 1 liquid) and at least two separate samples of each halogen salt were used in the experiments described.

The amount of halogen salt varied from 2 ml. of 3·24 *M* to 2 ml. of 0·05 *M*, each dilution being twice that of the previous one. Since the total volume in a tube was 13 ml. the concentrations of salt in the tubes varied from 0·5 to 0·008 *M*. With the alkaline earth halogen salts the dilutions were continued down to 0·001 *M*.

Results.

No firm coagulum was ever obtained on the addition of 1 ml. of 2 % pancreas substance to 10 ml. of milk and 2 ml. of water, and in practically every case the appearance of the mixture remained unaltered. With three samples of milk there was a fine granulation in the tube after keeping it at 37° for 30 minutes but never a definite coagulum. Probably these three samples of milk had a high content of calcium, chloride, or both.

Fluorides (Table I). The fluorides of Na, K and NH_4 at higher concentrations of salt (0·5–0·06 *M*) gave a definite coagulum with trypsin and milk. The acidity of the ammonium salt was sufficient to curdle the milk, and therefore, before using it in an experiment it was neutralised with anhydrous sodium carbonate.

Table I. *Fluorides.*

Concentration *M*	Time to produce clot		
	Na	K	NH_4
0·5	—	1 min. 41 secs.	4 mins. 13 secs.
0·25	—	2 mins. 55	4 10
0·125	4 mins. 18 secs.	3 48	4 10
0·06	4 27	4 37	5 18
0·03	No clot in 45 mins.	No clot in 1 hr. 10 mins.	No clot in 1 hr. 5 mins.
0·015	”	”	”
0·008	”	”	”
Standard	No clot in 1½ hrs.	No clot in 1 hr. 18 mins.	No clot in 2 hrs.

Below a concentration of 0·06 *M* the three fluorides did not give rise to a clot with milk and trypsin.

Chlorides (Table II). Li, Na, K and NH_4 chlorides all cause trypsin to coagulate milk, when added in concentrations of 0·5–0·008 *M*. With the chlorides of Mg, Ca and Ba, similar results were obtained, but the clotting time was shortest between 0·06 and 0·015 *M*; above and below these limits the action was marked, but less powerful.

Table II. *Chlorides.*

Concentration *M*	Time to produce clot			
	Li	Na	K	NH_4
0·5	1 min. 59 secs.	1 min. 26 secs.	1 min. 7 secs.	1 min. 1 sec.
0·25	1 54	1 17	1 8	0 53 secs.
0·125	1 53	1 25	1 13	0 57
0·06	2 mins. 14	1 44	1 29	1 12
0·03	2 53	2 mins. 15	2 mins. 8	1 33
0·015	3 52	2 57	2 59	2 mins. 22
0·008	5 2	4 27	4 29	4 38
Standard	No clot in 1 hr. 30 mins.	No clot in 2 hrs.	No clot in 1 hr. 30 mins.	No clot in 1 hr. 20 mins.

Concentration *M*	Time to produce clot		
	Mg	Ca	Ba
0·5	1 min. 38 secs.	1 min. 17 secs.	—
0·25	1 4	0 55	—
0·125	0 48	0 34	0 min. 53 secs.
0·06	0 38	0 26	0 37
0·03	0 38	0 26	0 29
0·015	0 49	0 36	0 37
0·008	1 8	0 57	0 58
0·004	1 37	1 24	1 25
0·002	2 mins. 25	2 mins. 5	2 mins. 20
0·001	4 10	3 40	4 21
Standard	No clot in 2 hrs.	No clot in 1 hr. 30 mins.	No clot in 1 hr. 45 mins.

The alkaline earth chlorides showed greater power to aid coagulation than did those of Li, Na, K and NH_4, but not to the extent previously noted with peptic clotting [Clifford, 1927].

There was no great difference between members of either group of chlorides in their capacity to aid tryptic coagulation, though $MgCl_2$ was rather less powerful in its action than $BaCl_2$ or $CaCl_2$.

Table III. *Bromides*.

Concentration	Time to produce clot			
M	Li	Na	K	NH_4
0·5	1 min. 2 secs.	1 min. 12 secs.	2 mins. 0 sec.	2 mins. 33 secs.
0·25	0 59	1 24	1 min. 59 secs.	2 5
0·125	1 10	1 34	2 mins. 23	2 3
0·06	1 25	1 53	2 52	2 25
0·03	1 56	2 mins. 29	3 54	2 59
0·015	2 mins. 58	4 37	5 1 sec.	3 48
0·008	No clot in 43 mins.	No clot in 40 mins.	6 3 secs.	4 45
Standard	No clot in 3 hrs.	No clot in 1 hr. 30 mins.	No clot in 2 hrs.	No clot in 1 hr. 15 mins.

Concentration	Time to produce clot		
M	Mg	Ca	Ba
0·5	5 mins. 4 secs.	1 min. 44 secs.	—
0·25	2 6	1 10	1 min. 10 secs.
0·125	1 min. 26	0 46	0 54
0·06	1 9	0 33	0 40
0·03	1 10	0 32	0 35
0·015	1 23	0 42	0 44
0·008	1 55	1 9	1 0 sec.
0·004	2 mins. 35	1 41	1 34 secs.
0·002	3 55	2 mins. 31	2 mins. 17
0·001	6 8	8 1 sec.	3 51
Standard	No clot in 2 hrs.	No clot in 2 hrs. 45 mins.	No clot in 2 hrs.

Table IV. *Iodides*.

Concentration	Time to produce clot			
M	Li	Na	K	NH_4
0·5	2 mins. 49 secs.	1 min. 30 secs.	1 min. 12 secs.	2 mins. 8 secs.
0·25	2 0 sec.	1 11	1 13	1 min. 17
0·125	2 17 secs.	1 22	1 24	1 30
0·06	2 47	1 42	1 48	1 49
0·03	3 22	2 mins. 19	2 mins. 37	2 mins. 16
0·015	4 25	3 28	4 15	3 15
0·008	No clot in 50 mins.	No clot in 1 hr.	No clot in 1 hr. 30 mins.	No clot in 35 mins.
Standard	No clot in 1 hr. 15 mins.	No clot in 2 hrs. 30 mins.	No clot in 2 hrs. 30 mins.	No clot in 1 hr. 30 mins.

Concentration	Time to produce clot		
M	Mg	Ca	Ba
0·5	1 min. 58 secs.	1 min. 32 secs.	1 min. 57 secs.
0·25	1 51	1 2	1 3
0·125	0 46	0 44	0 46
0·06	0 49	0 36	0 40
0·03	1 3	0 39	0 38
0·015	1 26	0 52	0 47
0·008	2 mins. 11	1 15	1 2
0·004	3 0 sec.	1 56	1 38
0·002	4 23 secs.	3 mins. 10	2 mins. 40
0·001	No clot in 30 mins.	No clot in 45 mins.	No clot in 1 hr. 25 mins.
Standard	No clot in 1 hr. 8 mins.	No clot in 2 hrs. 40 mins.	No clot in 1 hr. 5 mins.

Bromides (Table III). The results are similar to those obtained with chlorides, except that Li and Na bromides were inactive at a concentration of $0{\cdot}008M$.

Again the salts of Mg, Ca and Ba were less powerful at the highest concentration ($0{\cdot}5M$) and at the lower concentrations ($0{\cdot}008$–$0{\cdot}001M$), and $MgBr_2$ gave a less marked effect than $CaBr_2$ or $BaBr_2$.

Iodides (Table IV). Iodides also show a marked effect in enabling trypsin to clot milk. This action is not quite so powerful as that of the chlorides and bromides, since the salts of Li, Na, K and NH_4 show no effect at $0{\cdot}008M$, and those of Mg, Ca and Ba are inactive at $0{\cdot}001M$.

The slight inhibitory action of the high concentrations of the Mg, Ca and Ba salts is again marked, together with the less effective action of MgI_2 as compared with the other two iodides.

DISCUSSION.

All the halogen salts investigated enable trypsin to coagulate milk, irrespective of the cation. The reaction was least marked with the fluorides which showed no effect at concentrations below $0{\cdot}06M$. The chlorides and bromides of the alkaline earths caused trypsin and milk to form a coagulum in a concentration as low as $0{\cdot}001M$, but the iodides needed a concentration of $0{\cdot}002M$. At the highest concentrations used ($0{\cdot}5M$) the coagulating aid of the Li, Na, K and NH_4 halogen salts was as powerful as that of Mg, Ca and Ba, but the latter were more effective at concentrations below $0{\cdot}125M$.

From these results it seems that the acceleration of tryptic activity in clotting milk so often noticed when $CaCl_2$ is added is quite as much a function of the Cl as of the Ca, and that the addition of any other soluble halogen salt would be equally effective over a wide range of concentrations.

This accords with the theory of Kleiner and Tauber [1934, 2] who state that the clotting of milk by trypsin is due to a depression of proteolytic action, thus allowing the casein stage to show, since in a previous paper [Clifford, 1933] it has been shown that the halogen salts of Li, Na and K tend to depress the proteolytic action of trypsin.

This is not, however, a full explanation, since the fluorides are the greatest depressors of proteolytic action and are at the same time the least active in promoting milk coagulation, and the concentration of bromides at which no appreciable effect on proteolytic activity can be seen is considerably higher than that at which no effect on milk coagulation is demonstrable.

SUMMARY.

1. Milk does not give a firm clot with trypsin.

2. If the halogen salts of Li, Na, K, NH_4, Mg, Ca or Ba be added to milk it gives a firm clot with trypsin.

3. This coagulating action is least with fluorides (ceases at $0{\cdot}03M$).

4. The halogen salts of the alkaline earths are only slightly more efficient than those of Li, Na, K and NH_4.

5. At concentrations above $0{\cdot}25M$ the halogen salts of Mg, Ca and Ba are less effective than at concentrations between $0{\cdot}125$ and $0{\cdot}015M$.

6. The Mg salts are less powerful than those of Ca or Ba in promoting the clotting of milk by trypsin.

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REFERENCES.

Clifford (1927). *Biochem. J.* **21**, 544.
—— (1933). *Biochem. J.* **27**, 326.
—— (1934). *Biochem. J.* **28**, 418.
Kleiner and Tauber (1934, 1). *J. Biol. Chem.* **104**, 266.
—— —— (1934, 2). *J. Biol. Chem.* **104**, 271.
Mellanby (1912). *J. Physiol.* **45**, 345.
—— and Woolley (1912). *J. Physiol.* **45**, 370.
Seth (1924). *Biochem. J.* **18**, 1403.

CXXXIV. VITAMIN A-ACTIVE SUBSTANCES IN EGG-YOLK.

BY ALBERT EDWARD GILLAM AND ISIDOR MORRIS HEILBRON.

From the Chemistry Department, Manchester University.

(*Received March 16th, 1935.*)

ALTHOUGH biological experiments by numerous workers have shown that egg-yolk exhibits the growth-promoting properties usually associated with vitamin A, no wholly satisfactory explanation of the source of this activity has yet been advanced.

The presence of carotene in egg-yolk was first suggested by Willstätter and Escher [1911], being later confirmed by Kuhn and Brockmann [1932] in several varieties of hen's eggs, whilst the detection of vitamin A itself was claimed by Euler and Klussman [1932; 1933] using the antimony trichloride blue test as a criterion. As, however, these last authors were unable to detect with certainty the absorption band at 328 $m\mu$ characteristic of vitamin A, and as no details were given of any steps taken to remove xanthophylls before applying the antimony trichloride blue test, the results need confirmation. The detection of vitamin A in egg-yolk presents some difficulty on account of the smallness of the gross vitamin A activity (some of which is due to carotene), and the large amounts of lutein and zeaxanthin normally present in the yolk. As both of these latter substances absorb ultra-violet rays near 328 $m\mu$ and also give blue colours with antimony trichloride qualitatively similar to that given by vitamin A, the two main criteria for the vitamin are seriously interfered with unless a separation from the carotenoids is first obtained.

EXPERIMENTAL.

Vitamin A. In a preliminary experiment designed to detect the presence of vitamin A in egg-yolk the following procedure was tried. The gross unsaponifiable matter of thirty egg-yolks, after removal of sterols, was dissolved in light petroleum and about 10 mg. of a genuine vitamin A concentrate were added to one-half of the solution, whilst the other was left untouched. Both extracts were carefully treated with animal charcoal until the yellow colour of the carotenoids was just removed. Each solution was filtered and evaporated *in vacuo*, the residue being dissolved in chloroform and tested for vitamin A both by means of its ultra-violet absorption spectrum and by the antimony trichloride blue test. Whilst the genuine egg extract failed to give any positive data for the vitamin by either test, the one containing added vitamin A gave definite evidence of its presence. The conclusions to be drawn from this experiment are either that egg-yolk contains no vitamin A, or that, if present at all, the amount must be so small that a much finer method of separation is needed to detect it. With this object in view the following alternative procedure was adopted. The yolks of 60 eggs were mixed with anhydrous sodium sulphate and completely extracted with ether. After saponification of the extract with alcoholic potash the unsaponifiable portion was taken up in ether and the solvent-free residue crystallised from methyl alcohol. The sterols (18 g., M.P. 147°) were filtered off and the carotenoids transferred to light petroleum (B.P. 70–80°), the solution then being extracted 10–15 times with 90–92 % methyl alcohol. In this manner a light petroleum phase (A) containing only the carotenes [Kuhn and Brockmann, 1932] and a methyl alcoholic phase (B) containing the lutein, zeaxanthin and the major part of the vitamin A [Wolff *et al.*, 1930] were obtained. The constituents of B were again transferred to light petroleum (B.P. 40–60°), washed with water, dried and adsorbed on a column of calcium carbonate. The chromatogram was developed with petroleum-ether-benzene, a procedure whereby the xanthophylls would remain on the column while any vitamin A would pass through unadsorbed [Karrer and Schöpp, 1932]. The filtrate was evaporated *in vacuo* and the residue taken up in chloroform. On spectroscopic examination the solution exhibited a definite absorption band at 328 $m\mu$, whilst with antimony trichloride in chloroform a blue colour was obtained showing an obvious 618 $m\mu$ band together with the usual masked band at 583 $m\mu$ [Castle *et al.*, 1934]. The vitamin A in this particular batch of eggs was of the order of 0·05 mg. per 100 g. of yolk, and is probably lower than normal as the eggs were obtained from hens fed on a heavy maize ration, the yolks being paler than usual. In the yolks produced by hens on a richer grass diet approximately twice this amount of vitamin was found to be present. These experiments therefore provide definite confirmatory proof of the claim of Euler and Klussmann [1932; 1933] that vitamin A occurs in egg-yolk.

The carotene fraction. The dried light petroleum solution (A) obtained in the phase separation was passed through an alumina column and washed down with light petroleum. The resulting chromatogram indicated the presence of more than one carotenoid, usually as a broad yellow-brown zone near the top, a strong yellowish ring below and a weak pink-brown ring lower still. Each ring was separately eluted with methyl alcohol and spectroscopically examined, with the result that they were found to exhibit absorption maxima identical with each other and with those of authentic β-carotene. The slight difference in their adsorption behaviour suggests that the upper yellow-brown ring is due to kryptoxanthin [Kuhn and Grundmann, 1933] whilst the lower one is due to the typical

Table I. *Alumina adsorption of "carotene" fractions from the egg-yolks of maize-fed hens.*

	Zone 1	Zone 2	Zone 3
Colour of adsorbed pigment	Brownish yellow	Strong yellow-brown with bright yellow background	Several weak pink bands
Location in column	Very near the top	Lower down than 1	Below 2
Absorption spectrum of the pigment in light petroleum (B.P. 70–80°)	Indefinite	Bands at 478 and 450 $m\mu$	Bands at 480 and 451 $m\mu$
Phase tests:			
1. Petroleum and 90 % CH_3OH	Mainly alcohol-phasic	Wholly petroleum-phasic	Wholly petroleum-phasic
2. Petroleum and 95 % CH_3OH	Mainly alcohol-phasic	Partly alcohol-phasic	
Adsorption on $CaCO_3$	Held strongly	Weakly adsorbed as a yellow band	Unadsorbed
Nature of pigment	Xanthophylls *plus* oxidation products	Kryptoxanthin	Carotene
Relative amounts present	—	13	1

β-carotene. The presence of the former carotenoid in egg-yolk is almost certainly attributable to the ingestion of maize, which contains kryptoxanthin [Kuhn and Grundmann, 1934] in addition to zeaxanthin [Karrer *et al.*, 1929]. The presence of kryptoxanthin in egg-yolk was suggested as a possibility (although not submitted to test) by Brockmann and Völker [1934] to explain the relatively high values obtained by Euler and Klussmann [1933] for the carotene content of of egg-yolk.

In order to confirm, if possible, the occurrence of kryptoxanthin in egg-yolk we obtained eggs from hens specially fed on a rich maize diet, and extracted 60 yolks according to the above scheme. In this case the adsorption column exhibited three main coloured zones, the characteristics of which are given in Table I. These results and a comparison with authentic kryptoxanthin extracted from *Physalis* by the method of Kuhn and Grundmann [1933] confirm the presence of this pigment. A further comparison of the kryptoxanthin with lutein and β-carotene shows that its adsorption affinity is intermediate between those of these two substances, as its constitution and observed properties would lead one to expect.

Quantitative evaluation of the pigments in several batches of eggs gave the results shown in Table II.

Table II.

Batch no. ...	1	2	3	4	
No. of eggs	60	17	24	24	
Diet of hens	Heavy maize ration	Grass	No grass	Grass	
Total carotenoids	2·0	4·2	4·4	11·0	mg. per 100 g. yolk
Kryptoxanthin	0·19	0·14	0·013	0·17	
Carotene	0·015	0·02			
Vitamin A	Order 0·05	Order 0·10	—	—	
% kryptoxanthin in total carotenoids	9·5	3·3	—	—	

It is thus evident that the "carotene" fraction of egg-yolk consists largely of kryptoxanthin, which is remarkably similar in properties to β-carotene and easily mistaken for it.

SUMMARY.

Using chromatographic methods with spectroscopic control an examination has been made of the unsaponifiable matter of egg-yolk with a view to elucidating the cause of its growth-promoting activity. After a phase test separation of the carotene from the xanthophyll, followed by adsorption of the latter on calcium carbonate, it has been possible to obtain the vitamin A free from carotenoids and to confirm its presence both by the antimony trichloride blue test and by the characteristic absorption band at 328 $m\mu$. The petroleum-phasic carotenoids have also been examined and found to consist of kryptoxanthin and β-carotene. By feeding fowls on a diet rich in maize it has been found possible to increase the kryptoxanthin content of the resulting egg-yolks, although the final value for the content of this vitamin A-active carotenoid in egg-yolk is still very small (order 0·2 mg. per 100 g. yolk).

We are indebted to Dr S. J. Watson and Mr W. S. Ferguson, of Messrs Imperial Chemical Industries Ltd. Research Station, Jealotts Hill, for the collection and preparation of the egg samples.

REFERENCES.

Brockmann and Völker (1934). *Z. physiol. Chem.* **224**, 193.
Castle, Gillam, Heilbron and Thompson (1934). *Biochem. J.* **28**, 1702.
Euler and Klussmann (1932). *Z. physiol. Chem.* **208**, 50.
—— —— (1933). *Z. physiol. Chem.* **219**, 218.
Karrer, Salomon and Wehrli (1929). *Helv. Chim. Acta*, **12**, 790.
—— and Schöpp (1932). *Helv. Chim. Acta*, **15**, 745.
Kuhn and Brockmann (1932). *Z. physiol. Chem.* **206**, 41.
—— and Grundmann (1933). *Ber. deutsch. chem. Ges.* **66**, 1746.
—— —— (1934). *Ber. deutsch. chem. Ges.* **67**, 593.
Willstätter and Escher (1911). *Z. physiol. Chem.* **76**, 214.
Wolff, Overhoff and Eekelen (1930). *Deutsch. med. Woch.* **56**, 142.

CXXXV. THE STARCH AND RELATED POLYSACCHARIDES OF CERTAIN HARDWOODS.

I. THE PREPARATION AND PROPERTIES OF OAK AND WALNUT STARCH.

By WILLIAM GEORGE CAMPBELL.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Bucks.

(*Received March 18th, 1935.*)

So far as it can be ascertained the presence of starch in the parenchymatous cells of certain hardwoods has never been doubted. At the same time it is frequently admitted that the methods which have been used for its quantitative estimation are in the main unsatisfactory. Further it would appear that the polysaccharide itself has never actually been isolated from woody tissues and subjected to detailed chemical examination. In connection with the biological work [Fisher 1929], which has been in progress in this Laboratory for some years on the feeding habits of *Lyctus* powder-post beetles, it was shown [Campbell, 1929] that the larvae of this insect which bore in the sapwood of oak derive their nourishment from cell contents. Mer [1903] had already indicated that wood which is depleted of its starch is not attacked by *Lyctus canaliculatus* and *Anobium* sp. and this has recently been confirmed by Wilson [1933].

The importance of this biological work has rendered it desirable to enquire into the composition and properties of wood starch, and, if possible, to find reliable methods both of removing it from wood in commercial sizes and of estimating it quantitatively in experimental material. The substance is readily detected in woody tissues by means of iodine-potassium iodide solution, but in view of the absence of knowledge concerning its composition and properties a method has had to be developed for isolating it quantitatively from sawdust. The present communication is confined to a description of the general properties of wood starch and the method adopted for its isolation together with a record of preliminary evidence regarding its composition.

EXPERIMENTAL.

Microscopical examination has confirmed that the optical properties of the grains of starch in the parenchymatous cells of oak sapwood are similar to those of other starches. The grains are generally spherical or ovoid in shape and have a maximum diameter of 6–10 μ. Groups of grains suitable for microscopical examination were obtained free from the parent wood substance as follows. Small samples of dry sapwood sawdust were shaken with ether and, immediately the wood had settled, portions of the supernatant ether were placed on dry slides. When the ether evaporated large numbers of starch grains were found on the slides. In the dry condition the grains were opaque, but under reflected light their surfaces were brightly illuminated. On moistening with cold water the grains appeared suddenly to swell to a slight extent, whereupon they became

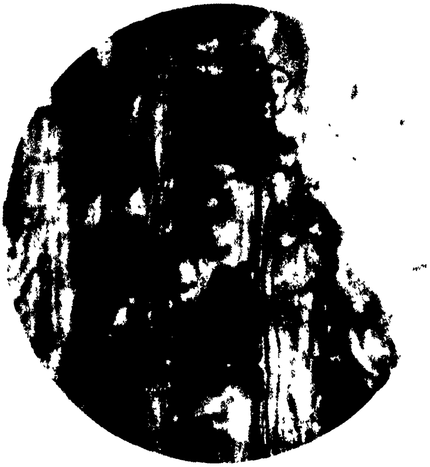

Fig. 1. Ray cell of oak sapwood (× 1000) containing starch grains lightly stained with iodine-potassium iodide solution.

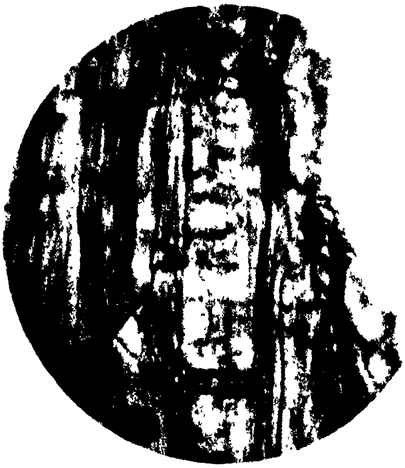

Fig. 2. Same cell after immersion in boiling water for 10 minutes.

Fig. 3. Same cell after final staining with iodine-potassium iodide solution.

translucent. Under crossed nicols a distinct black interference cross was discernible at approximately 600 diameters. The grains appeared to be structurally homogeneous and the presence of a hilum could not be detected.

Examination of wood starch in situ.

A small chip of oak sapwood was cut so as to expose a portion of a medullary ray. After moistening in cold water and staining lightly with a dilute iodine-potassium iodide solution a single cell containing upwards of 20 starch grains was brought into focus under the microscope. The individual grains were clearly discernible. After photographing, the chip was immersed in boiling water for 10 minutes and the same cell was brought into focus again. It appeared to be entirely devoid of starch, but on adding a drop of iodine solution the entire cavity of the cell and part of the walls stained a deep uniform blue. These results are illustrated in the accompanying series of photographs (Plate II, figs. 1–3) for assistance in the preparation of which the author is indebted to Messrs S. H. Clarke and W. R. Hutchins.

It would thus appear that the starch of oak wood occurs naturally in parenchymatous cells in the form of minute particles of a gel which is readily dispersed to a sol by hot water. It will be indicated later that under certain conditions it can be dispersed to some extent even by cold water. In the form of a sol the material diffuses out of the wood.

The isolation of wood starch.

The physical properties of the substance suggested that it might readily be removed from a wood meal by extraction with hot water, but early attempts to isolate it in a reasonably pure form from green oak sapwood were unsuccessful owing to contamination by soluble colouring matter, tannins, sugars *etc.* After experimenting with a number of different procedures the following proved to be satisfactory.

The green winter-felled sapwood of oak, previously found to contain starch by the iodine test, was converted to sawdust and dried as rapidly as possible in a current of air. When the moisture content reached approximately 12 % the material was screened and the portion which passed a 50-mesh and was retained by a 60-mesh screen was selected for extraction. Material of finer mesh was found to be unsatisfactory for reasons which will be apparent later. It was found by experiment that air-dry 50–60-mesh sapwood could be extracted with water at temperatures up to 60° in such a manner as to extract practically all the tannin and colouring matter and leave the starch grains apparently intact. Thereafter by extracting with water at 100° the starch could be removed with the minimum of contamination. The method used was as follows.

A series of 250 g. (air-dry) batches of the 50–60-mesh meal were extracted for 3 hours with constant stirring with 3 litres of water in 5-litre flasks maintained at 60° in a water-bath. After the wood had settled, the extract was siphoned off. The process was repeated usually four times until the final extract failed to give a tannin reaction with ferrous ammonium sulphate solution. Each extract was tested for starch with iodine-potassium iodide solution and it was found that while oak sapwood from one source gave no reaction in any of its 60° extracts a sample from a different source gave a definite starch reaction after the first extraction, but no reaction subsequently. So far, it has not been possible to explain this result for even after drying at 105° the second sample still yielded traces of starch at 60°. Examination of samples of the extracted wood meal under the microscope at this stage revealed that the ray cells still retained their starch in the form of grains.

The batches of wood meal were now subjected to a series of 3-hour extractions at 100° using 3 litres of water in each case. The resulting pale yellow colloidal solution, when cold, gave a pronounced starch reaction with iodine. Extraction was continued until the last extract gave no definite starch reaction. On the average four such extractions were sufficient. Microscopic examination of the wood at this stage revealed that all the starch grains had disappeared, but, on staining with iodine, cell walls and cavities here and there were coloured a pale blue. This was especially apparent in cells towards the interior of individual particles of wood. It was found that, if the 100° extractions were continued beyond this stage, another product, the colloidal solutions of which gave a royal purple colour with iodine was obtained. This will be referred to later.

The combined 100° extracts from groups of two batches of the original meal were concentrated *in vacuo* at 25–30° to a volume of 1·5 litres. The resulting dark brown colloidal solution was heated to approximately 70° in the water-bath, and acidified with 30 ml. of glacial acetic acid. An excess of hot 97 % ethyl alcohol was then added. The pale brown flocculent precipitate was allowed to settle and the supernatant alcoholic liquor containing tannin, colouring matter, soluble sugars *etc.*, was siphoned off. The product was then taken up in approximately 750 ml. of hot water and after the addition of a further 30 ml. of glacial acetic acid a further excess of hot alcohol was added. This operation was repeated 4-6 times until all colouring matter was removed, and the product was washed several times by decantation with alcohol until acid-free. It was then transferred to an extraction thimble and subjected to a series of 6-hour extractions with (1) absolute alcohol-dry benzene (1:2), (2) absolute alcohol and (3) dry ether. After removal of excess ether the product was transferred to a tared weighing bottle and the rest of the ether allowed to evaporate at room temperature, care being taken to stir frequently during this last operation. Final drying to constant weight was carried out in an air-oven at 105°. The final product was a pale pinkish buff impalpable powder which gave an intense blue coloration with iodine.

A sample of winter-felled English walnut sapwood was subjected to the same procedure as the oak and a final product of identical appearance was isolated. In the sample of walnut the starch was found to be confined to the outer layers of the sapwood, hence these only were converted. The actual yields obtained from typical extractions are recorded in Table I.

Table I. *Yields of starch from 50–60-mesh sapwood of winter-felled English oak and walnut.*

Results expressed as percentages by weight of original oven-dry wood meal.

	Oak sapwood					Walnut outer sapwood		
	1	2	3	4	5	1	2	3
Moisture content of original wood prior to extraction	11·39	11·25	11·13	11·15	11·14	9·37	8·87	8·87
Total loss in weight	10·62	9·93	10·65	Not determined	Not determined	8·94	10·60	9·17
Yield of starch	1·16	1·06	1·54	1·87	1·26	0·43	0·42	0·43
Average yield of starch	1·38					0·43		

The starch content of Lyctus *frass.*

It has already been shown [Campbell, 1929] that the frass of *Lyctus* powder-post beetles is so fine in texture that it readily passes a 120-mesh screen. A sample of frass was examined and found to contain traces of starch as revealed by the iodine test. A 200 g. (oven-dry) sample of each of 120-mesh oak sapwood and *Lyctus* frass obtained from a number of specimens of infested oak sapwood were extracted by the procedure outlined above (see Table II).

Table II. *Yields of starch from* Lyctus *frass and oak sapwood of the same particle size compared.*

	Sound oak sapwood 120-mesh	*Lyctus* frass from oak sapwood
Dry weight of original material	200 g.	200 g.
Total loss in weight	12·17 %	10·70 %
Yield of starch	0·90 %	0·28 %

The product in both cases was dark in colour and therefore probably contaminated to some extent by other substances. The preparation from material of such fine texture was much more difficult than from the 50–60-mesh material, the yield from the sound wood being lower than the average from 50–60-mesh material (Table I) although both were obtained from the same source. As would be expected the total loss in weight is greater in the fine material, owing to its state of division. It is noteworthy that total loss by exhaustive extraction with water is less in the frass than in the sound wood.

The physical and chemical properties of isolated wood starch.

On exposure to air the dried product from the sapwood of both oak and walnut absorbed moisture. After attaining an equilibrium moisture content of approximately 12 % there was no change in physical appearance. The starch was very slightly soluble in cold water, but more soluble in hot water to give a pale yellow colloidal solution. The solutions gave the characteristic blue colour with iodine which was discharged on heating and regenerated on cooling. There was no reduction of Fehling's solution until after hydrolysis with dilute HCl. When mixed with a little cold water on a glass plate the powder reverted to a brown-coloured gel which set hard on drying. In thin films the dried gel was almost colourless and transparent.

A sample of the powdered starch was dried to constant weight at 105°. After moistening with cold water it was again dried at 105° and yielded exactly the original weight.

It would thus appear that even in the solid form the material retains no more water at 105° than the powder.

Ultimate analyses of the product from the two different species of wood gave the following results.

Oak sapwood starch	Walnut sapwood starch
1. C, 43·95 %; H, 6·50 %	1. C, 44·29 %; H, 6·41 %
2. C, 44·11 %; H, 6·41 % N, 0·12 %	2. C, 43·96 %; H, 6·44 % N, 0·51 %
Mineral ash, trace	Mineral ash, 1·13 %

In view of the low yields of walnut material the following exploratory experiments were confined to the material from oak sapwood.

Specific rotation. A clear colloidal solution of the substance could only be obtained with difficulty. 0·4968 g. (oven-dry, 105°) was incorporated with 50 ml. of cold water and allowed to stand for several hours. The mixture was raised to 100°, but even after 4 hours at this temperature a considerable proportion of the starch remained undispersed. The mixture was transferred with a minimum of extra water to an alundum crucible of porosity R.A. 360 and filtered at the pump. The optical rotation of the pale yellow colloidal filtrate was measured after noting its volume at 20°. The undissolved residue was dried at 105° and weighed; c was found by difference: found $[\alpha]_D^{20^\circ} + 164{\cdot}3^\circ$ ($c = 0{\cdot}286$). A portion of the solution gave the characteristic blue colour with iodine and did not reduce Fehling's solution.

0·4968 g. (oven-dry 105°) of the substance dissolved in 75–80 ml. of 2 % NaOH in about 2 hours to give a dark brown colloidal solution. This was made up to 100 ml.: found $[\alpha]_D^{20^\circ} + 128{\cdot}8^\circ$ in a 25 mm. tube.

Action of takadiastase on oak sapwood starch. In the following experiments the actions of takadiastase on the soluble form of potato starch (B.D.H. preparation) and on oak sapwood starch were compared. The method used was similar to that used by Widdowson [1931]. Two samples of approximately 0·2 g. of each of the two substances were mixed with 100 ml. of water, heated in a water-bath for 30 minutes and cooled to room temperature. While the potato starch gave a clear solution the oak starch was not all dispersed. Part of it remained suspended as a gelatinous precipitate. 10 ml. of a 1 % solution of takadiastase were added to each sample and after acidifying with acetic acid and adding a little toluene the series was maintained at 38–40° in an air-oven. A blank consisting of 100 ml. of water and 10 ml. of 1 % takadiastase solution was subjected to the same treatment. At regular intervals a portion of one of each pair of samples was tested for starch with an iodine-potassium iodide solution. After 22 hours the potato starch solution gave a pale orange colour with iodine, but the wood starch solution gave a greenish colour. The suspended matter was stained a faint blue. A pale orange colour was only obtained with the wood starch solution after 64 hours at 38–40°. The remaining one of each pair of solutions and the blank were now removed from the oven, raised to boiling to remove toluene and destroy the enzyme and transferred to tared alundum filter crucibles (R.A. 360) with small quantities of water. There was a small residue in each case. This was washed with water and filtered at the pump. The residues were dried at 105° and weighed and the filtrates *plus* washings were examined optically after their volumes had been measured at 20° (Table III). In view of the slow rate of action of takadiastase in small concentrations on wood starch, the experiment was repeated using the modified technique advocated by Denny [1934]. Each sample weighed approximately 0·5 g. To each were added 25 ml. of water in a small conical flask and after heating at 100° for 30 minutes and cooling, 25 ml. of acetate buffer solution at p_H 4·5, 25 ml. of a 4 % takadiastase solution and a little toluene were added. The flasks were plugged with cotton-wool and incubated at 38–40°. A blank without starch was subjected to the same treatment. After 19 hours control solutions gave a pale orange colour with iodine. The suspended matter in the wood starch solution stained faintly blue. A small amount of suspended matter in the potato starch solution, consisting apparently of the ruptured envelopes of starch grains, also stained blue. The solutions were boiled for 1 minute and filtered, the residues being washed and dried and the filtrates examined as before (Table III). While in the first experiment the takadiastase blank had no detectable rotation, in the second experiment a marked dextrorotation was observed, so that in this latter case the angular readings for the hydrolysed starch solutions were corrected to exclude the amount of deflection due to takadiastase. The observation of Denny [1934] that ordinary pharmaceutical preparations of takadiastase contain reducing substances was confirmed. Even after boiling in the presence of the buffer solution as described above reducing substances were still present: found $[\alpha]_D^{20^\circ} + 53{\cdot}1^\circ$ ($c = 0{\cdot}89$ by difference). A separate sample of the buffer solution in water was found to be optically inactive.

Table III. *The effect of takadiastase on the soluble form of potato starch and oak sapwood starch respectively at 38–40°.*

	Potato starch			Oak sapwood starch		
	1	2		1	2	
Dry wt. of original sample, g.	0·2396	0·50	0·50	0·1812	0·4937	0·4937
Wt. of takadiastase taken, g.	0·10	1·00 *plus* acetate buffer at p_H 4·5		0·10	1·00 *plus* acetate buffer at p_H 4·5	
Duration of hydrolysis, hours	22	19	19	64	19	19
Dry wt. of total residue, g.	0·0045	0·0138	0·0130	0·0351	0·0591	0·0595
Dry wt. of residue from takadiastase control, g.	0·0012	0·0075	0·0075	0·0012	0·0075	0·0075
Dry wt. of starch converted in g.	0·2363 =98·62 %	0·4937 =98·74 %	0·4945 =98·82 %	0·1473 =81·28 %	0·4421 =89·54 %	0·4417 =89·44 %
Final volume of solution in ml. at 20°	144·00	112·0	114·0	128·00	110·5	127·5
Rotation of solution at 20°	+0·10°	+0·74°	+0·73°	+0·08°	+0·71°	+0·63°
Correction for rotation of takadiastase	0·00°	+0·47°	+0·47°	0·00°	+0·48°	+0·41°
Corrected rotation (α) of sugar solution	+0·10	+0·27	+0·26	+0·08	+0·23°	+0·22°
c g. per 100 ml. of sugar solution	0·16	0·45	0·43	0·12	0·40	0·35
$[\alpha]_D^{20°}$ of sugar solution	+62·5°	+60·0°	+60·5°	+66·6°	+62·5°	+62·8°

Table IV. *Determination of the starch contents of oak sapwood and of* Lyctus *frass from the same species by means of takadiastase.*

	Sound oak sapwood				*Lyctus* frass from oak sapwood	
	50–60-mesh (1)	50–60-mesh (2)	60-mesh (1)	60-mesh (2)	120-mesh (1)	120-mesh (2)
Dry weight of original sample, g.	1·8002	1·8002	1·7658	1·7658	1·8203	1·8203
Loss in weight, g.	0·1568	0·1611	0·1903	0·1930	0·0858	0·0871
Correction for residue from takadiastase, g.	0·0019	0·0019	0·0019	0·0019	0·0019	0·0019
Total loss of wood substance, g.	0·1587	0·1630	0·1922	0·1949	0·0877	0·0890
Average loss of wood controls, g.	0·1189	0·1189	0·1400	0·1400	0·0731	0·0731
Loss due to action of takadiastase, g.	0·0398	0·0451	0·0522	0·0549	0·0146	0·0159
Starch as % of oven-dry original sample	2·21	2·55	2·95	3·11	0·80	0·87

Determination of the starch content of wood by means of takadiastase.

A number of experiments were carried out in order to determine the effect of takadiastase on the starch in the parenchymatous cells of oak wood. 10 ml. of a 4 % solution of the enzyme at p_H 4·5 were found to remove all traces of the starch from parenchymatous cells of 2 g. (air-dry) of 60-mesh oak sapwood in 17 hours. The following method was used to determine the starch content of the wood and also of the *Lyctus* frass (Table II) from the same species.

Two 2 g. samples of each material were incorporated with 25 ml. of water in 250 ml. flasks and heated at 100° for 30 minutes. After cooling, 25 ml. of acetate buffer at p_H 4·5 and 10 ml. of 4 % takadiastase were added. A little toluene was now added, the flasks were plugged with cotton wool and incubated at 38–40° for 17 hours. Two further samples were subjected to the same treatment except that 10 ml. of water were substituted for takadiastase solution and a blank was introduced without the "wood flour." After incubation each flask was boiled for 1 minute and the contents filtered with 100 ml. of water. The residues were dried at 105° and weighed (Table IV).

Acid hydrolysis of oak starch.

11·7145 g. (oven-dry) of the starch were hydrolysed with 500 ml. of 2 % sulphuric acid at 100° for 6 hours. A dark brown precipitate formed at an early stage and at the end of the hydrolysis this was removed by filtration, washed with water till free from acid, dried at 105° and weighed. The hydrolysate was neutralised with hot barium hydroxide solution and barium carbonate [Wise *et al.*, 1933]. The inorganic barium salts were removed by filtration and the neutral sugar solution was concentrated *in vacuo* to a stage when a gummy precipitate formed. A small quantity of ethyl alcohol was added and the solution was filtered and concentrated *in vacuo* to a volume of 10–15 ml. Using the method of crystallisation from alcohol recommended by Anderson [1926] four crops of crystals were obtained. The combined yield was washed with alcohol and ether and dried to constant weight *in vacuo*. The yield was 7·2 g. which together with the unhydrolysed residue (6·14 %) and mineral ash (0·24 %) accounted for approximately 66 % of the starting material. The crystals had $[\alpha]^{20^\circ}_{''}$ +57° (in water), but no definite melting-point. A portion appeared to melt at 134–135° and the remainder at 175° with decomposition. The crystals obviously consisted of a mixture of sugars, but the rotation suggested that glucose was predominant. This was to some extent confirmed by the fact that under the conditions prescribed by Browne [1912, p. 349] a copious precipitate of glucosazone was obtained in 7 minutes.

Attempts to separate the components of the sugar mixture were unsuccessful, and it was therefore decided to examine the original substance for components other than anhydroglucose residues and to investigate the course of its hydrolysis with sulphuric acid polarimetrically.

On distillation with 12 % HCl the substance yielded small quantities of furfuraldehyde. When determined in a series of 1 g. samples by the Tollens method a yield of furfuraldehyde phloroglucide of 4·26 % (average of three determinations) was obtained. The substance also contained a trace of methoxyl. (Found OMe, 0·69 %, average of two determinations.) The Tollens naphthoresorcinol test for hexuronic acids [Browne, 1912, p. 384] gave a faint but positive result.

The amount of CO_2 given off by the substance on distillation with 12 % HCl was determined by the method of Dickson *et al.* [1930]. This when calculated as uronic acid anhydride gave a result of 4·65 % (average of six determinations). On the usual assumption [Lefèvre and Tollens, 1907] that uronic acid anhydride residues yield in this way one-third of their weight of furfuraldehyde phloroglucide it can be calculated that the original substance may have contained 2·22 % of pentosan (calculated as xylan). It will be seen later that this is unlikely.

Graded hydrolysis of oak starch with sulphuric acid.

A series of 0·5 g. (oven-dry) samples of the original substance was hydrolysed with 100 ml. of 2 % H_2SO_4 at 100° for periods ranging from ½ to 16 hours. At the end of each hydrolysis the unhydrolysed residue was filtered off and washed free from acid with water. The combined filtrate and washings was in each case cooled to 20° and examined for optical rotation before and after neutralisation with powdered barium carbonate (Table V).

Table V. *The influence of time of hydrolysis of oak sapwood starch with 2 % H_2SO_4 at 100° on the specific rotation of the hydrolysis products in acid and neutral solution respectively.*

Duration of hydrolysis in hours	Residue %	$[\alpha]_D^{20°}$ in acid solution	$[\alpha]_D^{20°}$ in neutral solution
0	0	—	+164·3°
½	6·92	+155·6°	+159·5°
1	6·22	+124·4°	+124·4°
2	5·07	+ 88·4°	+116·3°
4	4·12	+ 62·6°	+ 46·1°
6	4·69	+ 50·6°	+ 59·0°
8	4·33	+ 50·8°	+ 58·8° unchanged after 12 hours
12	4·23	+ 63·1°	+ 63·1°
16	5·47	+ 64·3°	+ 64·3° → + 55·7° after standing 24 hours

A specific rotation-time curve based on the results in Table V is given in Fig. 1.

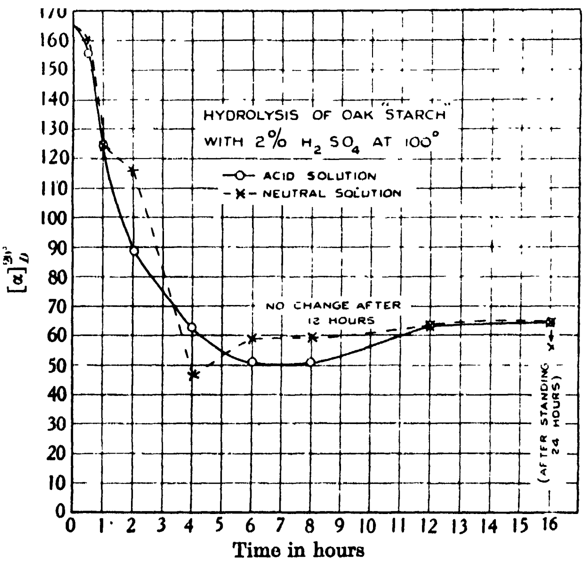

Fig. 1.

DISCUSSION.

From the experimental evidence cited above it would appear that a reliable method has been found for isolating the starch present in the parenchymatous cells of certain hardwoods in a reasonably high degree of purity. The essential feature of this method is that success or failure depends on the colloidal condition of the wood substance prior to extraction with water. The wood starch grain is readily dispersed in water. It can be removed from the green wood in the form of a sol by warm water. If, however, the moisture content of the wood substance as a whole be rapidly reduced to a value of 12 % or less the partially dehydrated starch grains become much more stable towards water. Advantage can be taken of this fact to remove the greater part of certain other water-soluble wood components in the first instance, so that the starch is ultimately obtained in a pure condition. It has already been shown that, during drying, wood substance as a whole undergoes certain changes which are reflected in the results of comparative analyses of green and dried material [Campbell and Booth, 1930; 1931; O'Dwyer, 1931], but this would appear to be the first direct evidence of a change in the colloidal condition of a minor wood component due to partial dehydration.

Although the experimental evidence suggests that this important wood component is intermediate in composition between starch and the known wood hemicelluloses, to designate it as other than "wood starch" at this stage would merely lead to confusion. There are some grounds for the belief that both oak and walnut sapwood contain the same substance, although in widely differing amounts, and, from experimental work at present in progress, it would appear that good yields can be obtained from the endosperm of the acorn.

The amounts of starch that can be isolated from the cell cavities of woods (Table I) are surprisingly small, and in the case of oak sapwood it can be seen by comparison of the results in Tables I and IV that estimation by means of takadiastase gives a much higher figure. In this latter method it can be seen that the average particle size of the wood sample has a definite influence on the result. Although it is freely admitted that the yields of starch obtained gravimetrically can only be approximately quantitative the results in Table IV might be taken to indicate a high degree of inefficiency in the method of extraction and precipitation which were adopted were it not for the fact that the cell walls of oak sapwood appear to contain a related polysaccharide. Reference has already been made to the observation that, if the extraction of oak sapwood with water at 100° be continued after all but the last traces of starch have been removed from the cavities of parenchymatous cells, another product, the colloidal solutions of which give a royal purple colour with iodine, can be isolated. For the present, little is known concerning this latter product apart from the fact that it is much more difficult to disperse with water than the starch proper. So far, an amount corresponding to 1·2 % of the dry weight of the original wood has been obtained from oak sapwood but more recently it has been found that the extractions of the wood with water at 100° must be prolonged for a total period of not less than 90 hours after the removal of the starch before the whole of this second product is obtained and it is expected that the yield will be greater. It has been found that part at least of this substance is converted into reducing sugars by takadiastase, but no quantitative data are yet available. The frequent assumption that takadiastase acts on substances other than the starch of the cell contents of wood is therefore apparently justified.

A significant chemical difference between potato starch and wood starch is brought out by the results in Table III. The action of takadiastase is much slower on the latter, but the specific rotations in the second experiment suggest that under optimum conditions the conversion products are the same in both cases. It must, however, be concluded that only approximately 90 % of wood starch can be composed of anhydroglucose units. The specific rotations suggest that the conversion products contain a proportion of maltose in the case of both the potato starch and wood starch. A further point which emerges from the results is that takadiastase itself even after heating to 100° for a short period contains reducing substances. The omission of any correction for the presence of these substances is as Denny [1934] has pointed out certain to lead to high results in the determination of starch when the amounts of the enzyme used are considerable. When the enzyme is used in the proportions suggested by Widdowson [1931], however, no appreciable error appears to be introduced (Table III). It has been seen above that the general accuracy of the determination of the starch content of wood, even by the modified takadiastase method used above (Table IV), cannot be assessed until more is known concerning the related polysaccharides of the cell walls.

Graded hydrolysis of the starch of oak sapwood.

The results in Table V which are illustrated graphically in Fig. 1 afford further important evidence concerning the composition of wood starch. The efficacy of the experimental procedure employed has recently been pointed out by O'Dwyer [1934], who has shown that a definite indication can thereby be obtained of the nature of the acid residues in oak hemicellulose A. It can therefore be deduced from the results (Table V) that, in the course of the hydrolysis of oak starch by dilute sulphuric acid at 100°, there is no appreciable splitting off of acid residues until the 2-hour stage is reached. Since the specific rotation of the hydrolysis products at this stage is higher in neutral than in acid solution, it is reasonable to conclude that the acid residues which are split off are combined with sugar residues in all probability to form an aldobionic acid. As the hydrolysis proceeds the main part of the starch molecule continues to break down to sugars and the aldobionic acid simultaneously breaks down to uronic acid and sugar until a stage is reached (4 hours) at which the predominance of uronic acid is sufficient to lower the specific rotation of the whole after neutralisation. Because of its relative instability towards mineral acids, however, the uronic acid is rapidly broken down to CO_2 and a pentose which ultimately yields furfuraldehyde, until at the 6- and 8-hour stages the aldobionic acid again predominates. No observations were made between 8 and 12 hours, but it is not unlikely that between these times the cycle of events already recorded was repeated. The specific rotations in acid and neutral solution finally become constant after 12 hours' hydrolysis and after 16 hours the specific rotation is reduced to 55·7° after mutarotation. There can be little doubt that the end-product is *d*-glucose.

An interesting dip in the curve (Fig. 1) for the acid solution is observed between 6 and 8 hours. This is no doubt explained by the formation of xylose from the uronic acid. Incidentally it can be deduced from this curve that after 6 hours' hydrolysis with 2 % sulphuric acid the hydrolysis products are likely to consist of a mixture of glucose, maltose, xylose, aldobionic acid and uronic acid, so that the failure, recorded above, to separate the components of such a mixture is largely explained. The specific rotation of +57° obtained for the products of hydrolysis of the large sample compares favourably with the value of +59° (Table V) for the small sample.

The data in Table V suggest strongly that the acid-building units in the original polysaccharide consist of an aldobionic rather than a uronic acid anhydride and that the anhydride in question is probably glucose-glucuronic anhydride. If the figures obtained for the amount of CO_2 given off on distillation with 12 % HCl be recalculated as aldobionic anhydride by means of the factor 7·7 an average result of 8·96 % of the dry weight of the original polysaccharide is obtained. This result, taken in conjunction with the results of the takadiastase experiments (Table III), is sufficient to account for 98·45 % of the original polysaccharide. Since, however, the yield of furfuraldehyde phloroglucide obtained from aldobionic acid anhydrides in general is not known, the actual percentage yield of the phloroglucide obtained above cannot at present be used to verify the position. No evidence has as yet been obtained to show whether the methoxyl groups determined are part of the molecule. For the present it can merely be stated that in all probability the starch of oak sapwood is composed of approximately 90 % of anhydroglucose residues, the remainder consisting of an aldobionic acid anhydride (probably glucose-glucuronic) which may or may not be partly methylated. The course of future work is obvious, and it will be pursued as soon as sufficient supplies of the polysaccharide are available.

Wood starch as a source of nourishment for the larvae of powder-post beetles.

There would appear to be little doubt that the polysaccharide which has been isolated is the substance to which Wilson [1933] has referred as the food of *Lyctus* larvae. The experimental evidence suggests that it contains approximately 90 % of available carbohydrate which can be converted into sugars by enzymic hydrolysis. The author is indebted to his colleague Mr E. A. Parkin for showing that the polysaccharide can also be converted into sugars by the gut enzymes of *Lyctus* larvae. Full details concerning this result will be published elsewhere in due course. If the nitrogen associated with the product is available in the form of protein the food value of the starch will thereby be enhanced although as yet there would appear to be no evidence of the presence of proteolytic enzymes in wood-eating insects [Mansour and Mansour-Bek, 1934]. It is noteworthy that *Lyctus* frass contains apparent starch which amounts to slightly less than one-third of the amount which can be isolated from sound wood of the same particle size (Tables II and IV). This result in itself might be taken to indicate the disappearance of the polysaccharide due to larval activity were it not for the fact that the starch content of woods is normally subject to seasonal variations. It may be argued that the presence of starch in the frass of this insect indicates that part of the wood actually comminuted by the larvae cannot be ingested. If this view be accepted it can readily be calculated from the data in Table II that the proportion of the total borings actually discarded might amount to one-third in some cases. The fact that *Lyctus* frass contains starch at all, however, indicates that the accurate determination of the starch content of a sufficient number of samples from a given species of wood, is certain to yield valuable information concerning the so-called immunity starch level of certain woods [Wilson, 1933].

Having regard to the small amounts of starch which have now been shown to occur in certain woods and also to the variations in composition known to exist between different samples of the same species of wood it can readily be appreciated why no significant differences in chemical composition could originally be detected between sound oak sapwood and *Lyctus* frass from the same species [Campbell, 1929]. Thus, despite recent criticisms [Wilson, 1933; Mansour and Mansour-Bek, 1934], the contention [Campbell, 1929], based on purely chemical evidence, that the source of larval nourishment is within the cells of the sapwood of oak and not in the cell wall substance, loses none of its force unless and until it be shown that the related cell wall polysaccharide referred to above is also a source of food. It may be repeated therefore that a complete solution of this question from the chemical point of view will only be attained when it is possible to feed the larvae of wood-boring insects on weighed samples of wood of known composition so that the losses in weight due to attack can be determined.

Another aspect of the feeding habits of *Lyctus* larvae merits consideration. It is asserted in some quarters [Fisher, 1928] that the insects only infest recently seasoned timber. It would be of interest to determine therefore whether, during the natural ageing of converted timber, the starch of the cell contents undergoes any chemical changes which might be likely to render it unsuitable to the insect. An early investigation of this question is contemplated.

The relationship of the starch of wood to certain other components.

The chief point of fundamental chemical importance which emerges from this exploratory examination of wood starch is that, until it can be proved to be other than a true chemical compound, this substance must be classified as an acid polysaccharide or hemicellulose. Hemicelluloses of this type have hitherto been considered to be of comparatively rare occurrence. The question naturally arises as to whether the starch of all "starch trees" is of the same composition. Much has been written on the chemistry of wood hemicelluloses and their relation to lignin, but the steps in the formation of these two wood components are still obscure. Buston [1934] has recently indicated after an extensive review of existing evidence that the hemicelluloses of lignified cell walls are probably not derived from pectin. The same author further postulates that because of their chemical composition such hemicelluloses must be derived from hexose polysaccharides. The composition of the wood starch which has now been isolated would appear not only to support this view but to provide a hitherto missing link between the hemicelluloses of the woody cell wall of certain species and the products of the chloroplasts of the leaf. The study of the chemistry of wood starch and the related polysaccharides in the cell walls is therefore being continued.

SUMMARY.

1. The starch present in the parenchymatous cells of certain woods is shown to resemble the known starches in some of its physical properties and to differ from them in others.

2. A method is described for the quantitative isolation of the substance in a high degree of purity from the sapwood of English oak and walnut.

3. Errors introduced into the determination of wood starch by means of takadiastase are discussed.

4. Wood starch is shown to be an acid polysaccharide approximately 90 % of which appears to be composed of anhydroglucose residues. It is suggested that the remainder consists of an aldobionic acid anhydride (probably glucose-glucuronic anhydride) which may or may not be partly methylated.

5. It is indicated that the cell walls of oak sapwood contain a polysaccharide which is chemically related to the starch of the cell contents.

6. Wood starch is considered to be a suitable source of nourishment for the larvae of *Lyctus* powder-post beetles.

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REFERENCES.

Anderson (1926). *J. Amer. Chem. Soc.* **48**, 3172.
Browne (1912). Handbook of sugar analysis. (Wiley, New York.)
Buston (1934). *Biochem. J.* **29**, 196.
Campbell (1929). *Biochem. J.* **23**, 1290.
—— and Booth (1930). *Biochem. J.* **24**, 641.
—— —— (1931). *Biochem. J.* **25**, 756.
Denny (1934). *Contrib. Boyce Thompson Inst.* **6**, 129.
Dickson, Otterson and Link (1930). *J. Amer. Chem. Soc.* **52**, 775.
Fisher (1928). *Forestry*, **2**, 40.
—— (1929). *Forest Products Research Bull.* No. 2. (H.M. Stationery Office.)
Lefèvre and Tollens (1907). *Ber. deutsch. chem. Ges.* **40**, 4513.
Mansour and Mansour-Bek (1934). *Biol. Rev.* **10**, 363.
Mer (1903). *Mém. Soc. Agric. France*, **140**, 233.
O'Dwyer (1931). *Biochem. J.* **25**, 2017.
—— (1934). *Biochem. J.* **28**, 2116.
Widdowson (1931). *Biochem. J.* **25**, 872.
Wilson (1933). *Ann. Appl. Biol.* **20**, 661.
Wise, Hamer and Peterson (1933). *Ind. Eng. Chem.* **25**, 184.

CXXXVI. A METHOD FOR THE MICRO-DETERMINATION OF MAGNESIUM.

By GEORGE CRUESS-CALLAGHAN.

From The Department of Physiology and Biochemistry, University College, Dublin.

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Two organic chemical reagents are convenient for the detection and estimation of small amounts of magnesium, *viz.* *op*-dihydroxyazo-*p*-nitrobenzene and 8-hydroxyquinoline. Kahn and Vieweg [1927] have described an accurate gravimetric determination of magnesium with the aid of 8-hydroxyquinoline. Hough and Ficklen [1930] have worked out the ranges over which this reagent can be used for the estimation of magnesium gravimetrically, volumetrically and colorimetrically. They used the gravimetric method for quantities from 10–1 mg. At the lower end of the range the error was about 10 %. Their colorimetric method had a range of 5–0·5 mg. but here again the percentage error at the lower end of the range was high. The volumetric method, involving titration with permanganate, was employed over a range 5–0·1 mg.; above 0·2 mg. the results were good but below this the method became inaccurate.

For physiological work an accurate method for determining quantities of magnesium down to 0·05 mg. (50γ) in ashed residues was desirable. This has been worked out and analyses down to 20γ are described in this communication. The useful application of a method of magnesium determination requires that calcium shall not interfere; it will be shown that 50γ of magnesium can be accurately determined in presence of more than 200γ of calcium.

The method involves formation of the magnesium complex of 8-hydroxyquinoline, its hydrolysis, and subsequent determination of free hydroxyquinoline, by bromination with excess bromine and determination of excess halogen. Hydroxyquinoline is brominated readily and quantitatively to 5:7-dibromo-8-hydroxyquinoline. Berg [1927] made use of this fact to determine 8-hydroxyquinoline in acid solution by titrating in a standard bromide-bromate solution until excess bromine was indicated by indigocarmine and determining this excess. Kolthoff [1927] employed the same method but used methyl red to indicate the excess bromine. Recently Harden and Wirjodihardjo [1934], using methyl red as an indicator, carried out a direct titration with a standard bromide-bromate solution. They estimated quantities of magnesium in soil from 24·01 to 5·55 mg. and gave the error as $-0{\cdot}18$ to $+0{\cdot}24$ mg. Modification of this method to deal with quantities of the order of 20–100γ was found by the author to be impracticable owing to the unsatisfactory nature of the end-point.

The method finally used for the analyses of quantities of magnesium down to 20γ is based on Berg's [1927] principle of adding a known excess of bromide-bromate solution and determining the residual bromine by titration with sodium thiosulphate after the addition of excess potassium iodide, starch being used as an indicator. Such iodimetric titrations of minute quantities of bromine can be accurately and conveniently carried out with the Conway micro-burette [1934] which delivers volumes of 0·1 ml. with the same percentage accuracy as 1·0 ml. is delivered from the standard 2 ml. Bang burette. The micro-technique involved required the introduction of several modifications in the procedure heretofore employed by workers using 8-hydroxyquinoline. These include the method of obtaining the complex free from the excess of 8-hydroxyquinoline, bromination without loss of bromine and the method of titration.

EXPERIMENTAL.

Formation of magnesium complex of 8-hydroxyquinoline. Three ml. or less of the fluid containing the magnesium to be estimated are introduced directly into a small centrifuge-tube of about 4 ml. capacity. A moderate excess of 8-hydroxyquinoline reagent (soln. 1) is added, and the fluid volume is brought up to approximately 3·5 ml. with distilled water. The centrifuge-tube is corked with a rubber stopper through which a capillary tube passes. This arrangement allows the air to escape on heating, and at the same time prevents the solution from overflowing owing to frothing. The centrifuge-tube is then placed in a small beaker (100 ml.) of water which comes about three-quarters of the way up the tube. The water is gradually heated to 90–95°, the magnesium 8-hydroxyquinoline complex separating out. The tube and its contents are maintained at the above temperature for a few minutes, then cooled and centrifuged for ten minutes. The supernatant liquid is syphoned off leaving only about 0·1 ml. This is diluted to about 3·5 ml. with approximately *N* ammonia solution. The sediment is disturbed to free it from any excess of the reagent and the tube is centrifuged again. As before the supernatant liquid is siphoned off. It may be shown that no disturbing excess of 8-hydroxyquinoline remains after the one washing.

Bromination of the equivalent amount of 8-hydroxyquinoline. To the washed precipitate a volume of standard bromide-bromate solution (soln. 2), sufficient to give an excess of bromine is added. When 100γ of magnesium are present 2·0 ml. of the bromide-bromate solution will be found to give a convenient excess, while 0·5 ml. will suffice when 20γ of magnesium are being determined. The volume is made up to approximately 2·5 ml. with distilled water and 1 ml. of 4*N* hydrochloric acid is added. This hydrolyses the complex, giving an equivalent of free hydroxyquinoline, and liberates bromine from the bromide-bromate solution. The centrifuge-tube is immediately stoppered and inverted for a moment while the sediment is disturbed by gentle tapping. The bromine is left in contact with the 8-hydroxyquinoline at room temperature for three minutes during which time the 8-hydroxyquinoline is converted quantitatively into 5 : 7-dibromo-8-hydroxyquinoline.

Estimation of excess bromine. A crystal of potassium iodide is added to the solution and the free iodine is titrated, using a micro-burette as described by Conway [1934] containing standard sodium thiosulphate solution (approx. *N*/40; see below) which is added until the colour of the iodine almost disappears. A drop of starch solution is then added and the titration is continued until the blue colour disappears. When the end-point is approached the rate of delivery of the burette may be slowed down, if it is inconveniently fast for accurate regulation, by reducing the delivery pressure of the burette as described by Conway [1934].

The colour change at the end-point can be seen quite clearly on adding about 0·002 ml. of *N*/40 thiosulphate solution which corresponds to about 2–3 graduations on the burette scale. This amount is in turn equivalent to approximately 0·15γ of magnesium. The end-point may therefore be regarded as satisfactory.

A blank determination for the whole procedure must be carried out simultaneously.

Solution 1. (*8-hydroxyquinoline reagent*): 1 g. of 8-hydroxyquinoline is dissolved in 89 ml. of absolute alcohol and 10 ml. of ammonia (sp. gr. 0·880) and 1 ml. of hydrochloric acid (sp. gr. 1·180) are added. 0·2 ml. of this reagent will suffice for the estimation of 100γ of magnesium. Corresponding amounts of the reagent may be used for less quantities.

Solution 2 (*bromide-bromate*): About 2·7 g. of potassium bromate and about 25 g. of potassium bromide are dissolved in 500 ml. of distilled water and the solution diluted 1 : 10. This will give approximately 1·6 mg. of bromine per ml. and is accurately standardised against the thiosulphate solution.

NOTE. Various methods of testing for an excess of bromine after bromination were tried. The most satisfactory found was to remove a small platinum loopful of the solution and bring it into contact with potassium iodide solution containing starch on a white background. A blue colour indicates excess of bromine.

Solution 3 (*sodium thiosulphate*): Using a micro-burette with a graduated tube of about one mm. bore, approximately $N/40$ thiosulphate will be found convenient. This is made up by dissolving about 6 g. of $Na_2S_2O_3, 5H_2O$ in one litre of distilled water. The solution may be standardised against $N/200$ potassium iodate solution (0·1775 g. KIO_3 per litre). One ml. of this is introduced into a centrifuge-tube, using an Ostwald pipette. A crystal of potassium iodide and a few drops of $N/10$ sulphuric acid are added. The titration is carried out in the usual way, using a drop of starch solution towards the end-point.

The absolute normality of the thiosulphate solution is not determined, but it is standardised in terms of length of fluid column in the graduated tube of the Conway burette, corresponding to 1 mg. or to 1 γ of bromine.

RESULTS.

The above method was used in the micro-determination of magnesium in standard solutions of magnesium sulphate containing about 6 g. of pure crystalline $MgSO_4, 7H_2O$ in 100 ml. of distilled water. The exact quantity of magnesium in 10 ml. of this solution is determined in duplicate by precipitating as magnesium ammonium phosphate, igniting and weighing as magnesium pyrophosphate. The solution thus made up and standardised for its exact magnesium content is suitably diluted and the requisite volumes used in the analyses.

Table I shows the results of analyses which were carried out using the method described with varying amounts of standard magnesium solution. It will be seen that down to 20γ the method may be relied on to give a result of an individual determination to within 10 % of the theoretical quantity.

Table I.

Theoretical amount of mg. γ	Analyses. Mean value γ	Number of analyses	% deviation of mean value from theoretical	Coefficient of variation of individual determinations
114·2	120·4	6	+5·0	1·2
76·1	74·3	6	−2·4	2·9
50·8	53·5	7	+5·3	3·9
25·4	24·2	6	−4·7	4·7
19·0	20·2	6	+6·3	4·3
12·7	13·2	6	+7·9	10·3
5·1	5·5	6	+7·8	6·1
3·8	3·3	5	+13·2	14·5
Blank	1·5	13	—	27·5

Table II gives individual analyses carried out on 25·4γ of magnesium. The percentage deviation of each analysis from the mean is given and it will be seen that the greatest is $-7·0$. The percentage deviation of the mean value of the six analyses from the theoretical is $-4·7$.

Table II. *Six estimations of 25·4γ Mg.*

Volume of bromide-bromate soln. used (ml.)	Thiosulphate soln. Divisions on micro-burette scale	Mg as estimated. Corrected for blank* (γ)	Deviation from mean γ	% deviation from mean
0·7	10·87	23·6	−0·6	−2·5
0·6	5·89	25·3	+1·1	+4·5
0·6	6·82	23·9	−0·3	−1·2
0·6	6·24	24·8	+0·6	+2·5
0·6	7·73	22·5	−1·7	−7·0
0·6	5·84	25·4	+1·2	+5·0

* Corrected by subtracting blank value = 1·5γ.

1 division (100 small graduations on) microburette scale used = 0·04435 ml.

1 ml. bromide-bromate solution is equivalent to 1·58 mg. Br.

1 division (100 small graduations) on micro-burette scale used, containing approximately $N/80$ thiosulphate solution is equivalent to 0·041 mg. Br. (A weaker thiosulphate solution was used here than was subsequently considered more generally suitable.)

Mean value of the six analyses of 25·4γ of magnesium is 24·2γ.

Six determinations of the blank value were carried out, using 0·1 ml. of 8-hydroxyquinoline and seven estimations using 0·2 ml. The mean values of these two series were practically the same (1·5γ) though individual values varied from 1·0γ to 2·5γ. This blank value is due to the glass and it will be seen that the variation between the individual blanks (up to 1·0γ from the mean) may account to a large extent for the high deviation of individual analyses where quantities less than 20γ are determined.

Determination of magnesium in the presence of calcium.

Table III gives the results of determinations of magnesium in presence of varying amounts of calcium.

Table III.

Wt. of calcium added γ	Mean value of four analyses γ	Apparent increase of magnesium due to presence of calcium γ
0	53·5	—
10	54·4	0·9
25	54·5	1·0
80	53·9	0·4
100	53·1	−0·4
200	55·8	2·3

In each case four analyses were carried out and the mean value of these determinations is given in column 2 of Table III. From these figures it will be seen that the analysis of 50γ of magnesium in 3·5 ml. may be carried out without any appreciable error due to the presence of four times this quantity of calcium.

SUMMARY.

1. A method is described whereby quantities of magnesium down to 20γ in 3 ml. or less can be reliably determined in standard solutions or in solutions from ashed residues.

2. Analyses were carried out on known quantities of magnesium varying from 114·2 down to 3·8γ. The results of these analyses are given and the figures show that over a range 114 to 20γ the coefficient of variation of the individual determination is 1·2 at the higher and 4·3 at the lower level. An individual

analysis therefore down to 20γ of magnesium may be relied on to within 10 % of the theoretical quantity. With this amount of magnesium the error of estimate is chiefly due to the glass of the centrifuge-tubes used.

3. It has been shown that, by the method described, 50γ of magnesium in 3·5 ml. can be determined in the presence of four times as much calcium without any increased error due to the calcium.

REFERENCES.

Berg (1927). *Z. anal. Chem.* **71**, 23.
Conway (1934). *Biochem. J.* **28**, 283.
Harden and Wirjodihardjo (1934). *Chem. Weekblad.* **31**, 662.
Hough and Ficklen (1930). *J. Amer. Chem. Soc.* **52**, 4752.
Kahn and Vieweg (1927). *Z. anal. Chem.* **71**, 122.
Kolthoff (1927). *Chem. Weekblad.* **24**, 254.

CXXXVII. THE FORMATION OF HYPONITROUS ACID AS AN INTERMEDIATE COMPOUND IN THE BIOLOGICAL OR PHOTOCHEMICAL OXIDATION OF AMMONIA TO NITROUS ACID.

II. MICROBIOLOGICAL OXIDATION[1].

By ALEXANDER STEVEN CORBET.

From the I.C.I. Agricultural Research Station,
Jealotts Hill, Bracknell, Berks.

(*Received March 27th, 1935.*)

The present investigation is concerned with the oxidation of ammonium salts to nitric acid in soil. In Part I [Corbet, 1934] the chemical aspects of the subject were dealt with, and in this paper the reactions effected by bacterial agency are considered.

For work on the biological oxidation of ammonia, aqueous solutions of ammonium sulphate containing small amounts of nutrient salts were inoculated with nitrifying organisms from soil. No particular attention was paid to the species of organisms present, but difficulty was experienced frequently in obtaining a culture capable of effecting complete and rapid nitrification under experimental conditions. On many occasions inoculation of Beesley's medium with small quantities of soil from arable land failed to produce more than traces of nitrite, even after prolonged incubation. With a few soil samples obtained at Jealotts Hill, nitrite production in Beesley's medium was strong and rapid but usually less than 5 % of the ammonium salt was decomposed after several weeks' incubation at 32°.

In one instance only did nitrification proceed beyond the stage of nitrite formation, and no attempt was made to ensure nitrate production, as the present investigation was concerned primarily with the intermediate compounds formed during the oxidation of ammonia to nitrous acid.

The formation of intermediate compounds.

It has been found in this investigation that the microbiological oxidation of ammonium (as sulphate) can proceed by the following stages:

$$NH_3 \rightarrow NH_2OH \rightarrow H_2N_2O_2 \rightarrow HNO_2 \rightarrow HNO_3.$$

There appears to be no doubt that hydroxylamine occurs as an intermediate compound during the early stages of nitrification, although on account of the reaction of the medium it is present in quantity too small to admit of accurate estimation. In some series of experiments, when nitrification had begun, this compound was detected by its reducing action on Fehling's solution and on iron alum, but it was never present when appreciable quantities of nitrite had accumulated.

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[1] Some aspects of the work described in this paper were considered in a paper read at the Annual General Meeting of the Association of Applied Biologists held in London on 15th February 1935.

It was shown experimentally that cultures of soil micro-organisms able to convert ammonium (as sulphate) into nitrite transformed hydroxylamine into nitrite under the same conditions. In one such experiment, 95 % of the nitrogen originally present as hydroxylamine disappeared, leaving the residue in the form of nitrite. It was evident that the hydroxylamine had undergone oxidation to nitrite, and obviously the resulting hydroxylamine nitrite had broken down to nitrous oxide and water.

Although hydroxylamine was detected as an intermediate product on occasions, it could not accumulate in the culture flasks since it becomes progressively less stable with increasing alkalinity of the medium. The data given in Table V show that some decomposition of hydroxylamine takes place at p_H 4·6, while at higher values the breakdown is rapid. In presence of nitrite, hydroxylamine is unstable whatever be the reaction of the medium (Table VI). Addition of nitrite is an easy and effective means of removing hydroxylamine from solution, and advantage was taken of this fact in the analytical procedure.

Hyponitrous acid was frequently detected as an intermediate compound during the early stages of nitrification and, with some cultures of soil micro-organisms, it was present in quantity.

In one series of experiments with ammonium sulphate, a considerable portion of the nitrogen known to be present could not be accounted for as ammonia, hydroxylamine, nitrous or nitric acid, and this deficit was greatest between the disappearance of ammonia and the formation of nitrite. It was concluded that the nitrogen was present in the form of hyponitrite.

This experiment was carried out in the early stages of the investigation, before a satisfactory method of estimating hyponitrous acid had been elaborated. Later work however fully confirmed the tentative conclusion. This substance was often detected during the early stages of nitrification, but in one series of experiments it constituted an important intermediate substance in a biological reaction, whereby the nitrogen in ammonium sulphate was finally lost, presumably in a gaseous form.

Calcium hyponitrite is stable in aqueous solution and in presence of nitrite, but on warming it decomposes and the nitrogen is lost in gaseous form. Although no chemical mechanism is known whereby hyponitrites are oxidised to nitrite, when an aqueous solution of calcium hyponitrite was inoculated with soil micro-organisms, there was an accumulation of nitrite and no gas evolution took place.

Attention has already been drawn [Corbet, 1934] to the autoxidation of nitrous acid which takes place rapidly in media of $p_H < 5$.

DISCUSSION.

In Tables I–IV and Figs. 1–3 are given some of the results obtained during the present investigation. It was found preferable to work with a number of separate culture flasks, since nitrification was neither vigorous nor rapid when a large bulk of liquid medium was inoculated and aliquot portions were withdrawn at intervals, and so in some instances the experimental points do not lie on a smooth curve.

It will be seen that while hydroxylamine was hardly ever present in measurable amount, hyponitrous acid constituted an important intermediate compound in the first and fourth series of experiments. Since the mutual presence of hydroxylamine and nitrite results in loss of nitrogen, it is not surprising that these two compounds do not co-exist at any stage during the biological nitrification of Beesley's medium. The fact that hydroxylamine and hyponitrous acid occur during the initial stages of the decomposition and are absent later, when appreciable amounts of ammonia still remain to be oxidised, is discussed on p. 1089.

Table I. *First series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution							
Days after inoculation	0	8	14	18	24	30	42	76
Nitrogen present as:								
Ammonia	19·6	15·0	4·7	10·7	9·0	7·8	8·8	0·1
Nitrous acid	.	1·7	3·0	7·1	9·8	11·0	10·3	19·6
Total accounted for	19·6	16·7	7·7	17·8	18·8	18·8	19·1	19·7
Deficit	.	2·9	11·9	1·8	0·8	0·8	0·5	.
Hyponitrous acid-N present according to titration with $KMnO_4$	.	3·0	11·4	3·6	0·8	0·8	0·3	.

No nitrate was found in any of the culture flasks. These results are expressed graphically in Fig. 1. There was, of course, no actual regeneration of ammonia: the apparent increase in ammonia-nitrogen on the 18th day is attributable to the fact that the reaction did not proceed at the same speed in all the culture flasks.

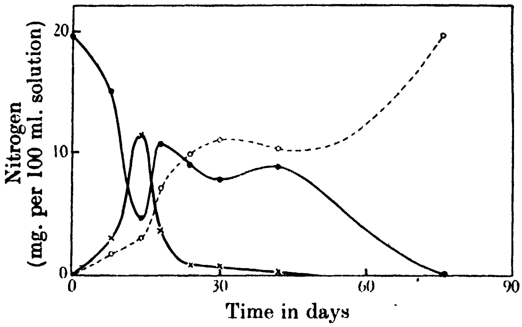

Fig. 1. First series of nitrification experiments.
•—• Ammonia-N. o- - -o Nitrous-N. x — x Hyponitrous-N.

Table II. *Second series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution							
Days after inoculation:	0	11	15	21	27	33	40	48
Nitrogen present as:								
Ammonia	19·2	18·8	18·5	18·0	18·5	18·9	18·3	18·6
Hydroxylamine	.	.	.	0·1	.	.	.	.
Nitrous acid	.	0·4	0·2	0·7	0·3	0·2	0·4	0·6
Total accounted for	19·2	19·2	18·7	18·8	18·8	19·1	18·7	19·2
Deficit	.	0·0	0·5	0·4	0·4	0·1	0·5	0·0

No nitrate was detected in this series of experiments.

Subcultures from this experiment and many other cultures of soil micro-organisms gave similar results. In one experiment, in which the course of nitrification was followed by measurement of the diminution in pressure, it was found that biological activity ceased when a small proportion of the ammonia had been oxidised.

The culture flasks were incubated at 32°, and qualitative experiments showed that the course of nitrification was similar when incubation took place at 4°.

Table III. *Third series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution										
Days after inoculation	0	3	5	7	10	13	17	19	24	28	42
Nitrogen present as:											
Ammonia	19·2	18·5	18·2	18·7	17·8	15·9	11·0	11·3	7·2	2·1	3·5
Nitrous acid	.	0·0	0·4	0·3	1·0	2·7	7·1	6·8	9·1	16·7	15·3
Total accounted for	19·2	18·5	18·6	19·0	18·8	18·6	18·1	18·1	16·3*	18·8	18·8
Deficit	.	0·7	0·6	0·2	0·4	0·6	1·1	1·1	2·9	0·4	0·4
Qualitative tests:											
Hydroxylamine	–	+	–	+	–	+	–	–	–	–	–
Hyponitrous acid	–	+	+	+	+	+	–	–	–	–	–
Nitric acid	–	–	–	–	–	–	–	–	–	–	–

* No satisfactory explanation of this low value was discovered.

A subculture from the above experiment gave similar results.

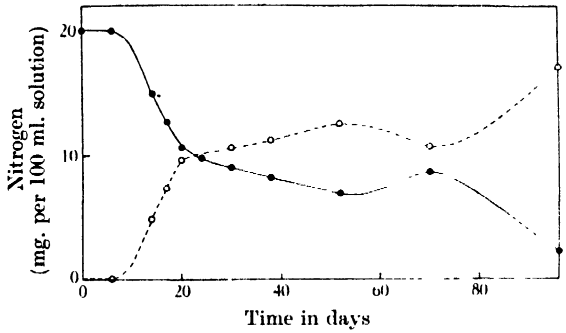

Fig. 2. Third series of nitrification experiments.
●—● Ammonia-N. o- - -o Nitrous-N.

In Part I attention was drawn to work carried out by Beesley [1914] on the biological oxidation of ammonium salts, in which, at one stage during an experiment, a considerable proportion of the nitrogen known to be present was unaccounted for, although finally it appeared in the form of nitrate. That the missing nitrogen was present partly, if not entirely, as calcium hyponitrite there can now be little doubt. The results obtained by Beesley were closely paralleled in the first series of nitrification experiments performed by the present writer (Table I and Fig. 1). Although in this case the hyponitrite was not estimated gasometrically, the qualitative tests leave no room for doubt that the missing nitrogen existed at least partly, and probably entirely, in the form of calcium hyponitrite. Chemical evidence has been adduced to show that there could have been no accumulation of hydroxylamine under the circumstances.

The evidence available strongly suggests that nitrification may be effected by a number of different species or strains of bacteria. It is unlikely that cultivation in the laboratory has produced drastic changes in the enzymic activities of the micro-organisms, for subcultures invariably gave the same type of reaction as the original cultures. The only way in which artificial conditions appeared responsible for any change was that in the type detailed in the third series of experiments production of hydroxylamine and hyponitrous acid was definitely associated with the logarithmic increase phase of growth, although repeated subculturing reduced these intermediates to vanishing point.

Table IV. *Fourth series of nitrification experiments.*

	Nitrogen present in mg. per 100 ml. solution									
Days after inoculation	0	2	3	6	8	10	13	17	35*	55
Nitrogen present as:										
Ammonia	19·8	18·0	18·0	16·5	15·8	15·0	14·0	14·0	10·2	5·9
Hyponitrous acid	.	1·4	n.d.	2·4	n.d.	1·7	2·1	n.d.	n.d.	0·7
Nitrous acid	.	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Total accounted for	19·8	19·4	.	18·9	.	16·7	16·1	.	.	6·6
Deficit	.	0·4	.	0·9	.	3·1	3·7	.	.	13·2
Qualitative tests:										
Hydroxylamine	−	−	−	−	−	−	−	−	−	−
Hyponitrous acid	−	+	+	+	+	+	+	+	+	+
Nitric acid	−	−	−	−	−	−	−	−	−	−

n.d. means not determined.

* Determination of the total nitrogen by the Kjeldahl method (using selenium as a catalyst) in a parallel flask on the 35th day gave a value of 10·0 mg. nitrogen per 100 ml. solution.

The results are plotted in Fig. 3. Subcultures gave precisely similar results. In one series, a solution which contained initially 20·3 mg. NH_3-N per 100 ml. solution contained 4·4 mg. NH_3-N and 1·0 mg. $H_2N_2O_2$-N and no hydroxylamine or nitrate and only a trace of nitrite after 81 days.

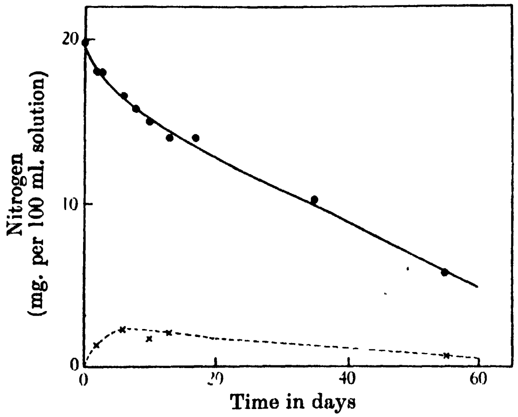

Fig. 3. Fourth series of nitrification experiments.
●—● Ammonia-N. ×---× Hyponitrous-N.

The most interesting type of biological nitrification encountered is that detailed in the fourth series of experiments (Table IV and Fig. 3) with a culture obtained from soil under grass which had been treated with "nitrochalk" some 6 months previously. This particular soil was selected since it repeatedly gave a strong positive reaction for hyponitrous acid with the resorcinol and potassium periodate test. With this culture, disappearance of ammonia was rapid, and after about 10 % of the nitrogen had accumulated in the form of hyponitrite, the proportion of the latter remained constant throughout the experiment. Presumably the missing nitrogen escaped in gaseous form.

Nitrification in soil.

It appears that the biological production of nitrite from ammonia in soil takes place in a variety of ways. The repeated failures to isolate nitrate-forming bacteria from soil suggest that *Nitrobacter* is by no means so widely distributed as the nitrite-forming organisms, and that the further oxidation of nitrite to nitrate may be attributable to autoxidation in acid media.

Evidence obtained from laboratory experiments indicates that hyponitrites may occur in soils, at least under some conditions, and tests at Jealotts Hill have shown that small amounts of hyponitrite are in fact present in certain soils. On the other hand, evidence has been advanced by Gopala Rao and Dhar [1931; Gopala Rao, 1934] and by the writer, showing that in exposed soils in the tropics, nitrification is, at least partly, a chemical process. Nitrite formation may be effected by a photochemical reaction and, to a lesser extent, by alternate wetting and drying-out of soils.

The depth to which ultraviolet light can penetrate the soil is an important matter in this connection in the tropics. It is believed that photonitrification of ammonium salts is effected by light of the wave-length 3100–2900 Å. The mercury arc emits radiations down to 1800 Å., while the solar spectrum does not penetrate into ultraviolet regions beyond 2900 Å. It was found in the laboratory that whilst light from a mercury vapour lamp effected nitrite-formation in a silica gel impregnated with ammonium sulphate to a depth of several inches, no liberation of iodine from potassium iodide occurred through 0·25 inch of moist or dry soil. Nevertheless, the question of penetration of soil by chemically active radiations from sunlight cannot be readily dismissed: Hoerlin [1934] states that the ultraviolet radiation in equatorial latitudes is of different intensity and composition from that in temperate regions. Many of the chemical reactions caused by ultraviolet light are effected also by the action of hydrogen peroxide (*e.g.* liberation of iodine from potassium iodide, nitrite production from ammonia [Weith and Weber, 1874] and the formation of a pink substance from ammonium thiocyanate), and this may have some bearing on the question of the depth of photonitrification in exposed tropical soils which are wetted daily. It must also be borne in mind that ammonium salts often occur "free" in such soils, not being adsorbed by the soil colloids, and in equatorial latitudes the top soil layer is much more shallow than in temperate regions.

Once nitrite is formed in the acid soils of equatorial countries, the further oxidation to nitrate must be largely effected by chemical means.

The criticisms advanced by Fraps and Sterges [1935] against the work of Dhar and Gopala Rao are without substance, since in their experiments soil was exposed to the sun in pyrex beakers covered with glass. The writer has confirmed that no ultraviolet radiations of wave-length capable of oxidising ammonium salts to nitrite can penetrate these materials.

Denitrification in sunlight.

Recently Dhar [1934] has sought to explain the losses of nitrogen from soil, known to take place under certain conditions in presence of readily oxidisable organic matter, as a result of the decomposition of ammonium nitrite in sunlight. Dhar has found that when mixed with sterilised or unsterilised soil, or in presence of a photosensitiser such as titania, zinc oxide or ferric oxide, ammonium nitrite undergoes decomposition with liberation of free nitrogen: since nitrite is an intermediate in nitrification, it is supposed that ammonium nitrite is formed and then disrupted under the action of light.

The present writer found that a solution of ammonium chloride and sodium nitrite in stoichiometrical proportions (p_H 6·8) showed no decomposition in the dark during a period of 3 months, and only slight losses of ammonia occurred, the nitrite content remaining unchanged, after 87 hours' irradiation by the mercury arc (Table VII). In presence of freshly-ignited titania however, both in sunlight and under the mercury arc, small quantities of gas were liberated. In view of the fact that nitrite is so readily transformed into nitrate in acid soils, it seems doubtful if any considerable losses from soil can occur in this way except, possibly, in arid regions where nitrite accumulation takes place.

While discussing nitrogen losses from the soil, attention may be drawn to the importance of the results of the fourth series of nitrification experiments.

Jenny [1929] has shown that, in general, the nitrogen content of the soil is a function of the mean annual temperature and humidity, and thus for any given mean annual temperature and humidity, the potential nitrogen content of the soil has a maximum value. Apart from this it is evident that the nitrogen content of the soil cannot be raised indefinitely, and that some means must exist of ridding the soil of surplus nitrogen when the potential nitrogen content is exceeded as, for instance, in heavy applications of nitrogenous fertilisers. The nature of this mechanism is obscure, and it may be biological or chemical in character.

In the fourth series of nitrification experiments described in this paper, a loss of nitrogen followed the conversion of ammonium sulphate into hyponitrite, and the only plausible explanation is that the missing nitrogen has been released in gaseous form. This may be an example of a biological mechanism effecting the escape of excessive amounts of nitrogen from the soil. In the present case however the detection and estimation of gaseous forms of nitrogen released by bacterial agency is difficult, since the reaction can proceed only in presence of adequate supplies of oxygen.

EXPERIMENTAL.

The decomposition of hydroxylamine.

Solutions of hydroxylamine hydrochloride were prepared, and the reaction was adjusted by addition of sulphuric acid or sodium hydroxide; the p_H values were determined colorimetrically. The solutions were preserved in the dark in stoppered flasks, and the hydroxylamine content was determined by boiling with ferric alum and sulphuric acid, followed by titration of the reduced iron with potassium permanganate.

Table V. *Loss of hydroxylamine from aqueous solution at different* p_H *values.*

Initial conc. 0·0073 g. NH_2OH per 100 ml. Temperature 32°.

	% Loss				
p_H of solution	After 1 day	After 7 days	After 14 days	After 23 days	After 38 days
2·2	0·0	0·0	0·0	0·0	0·0
4·6	1·0	1·7	9·8	—	—
5·9	46·1	46·9	59·4	61·1	60·2
8·4	46·1*	66·3	75·2	81·7	86·9

* Determination of other forms of nitrogen in this solution gave NH_3-N nil; nitrite-N, trace; nitrate-N, 0·001 %.

It is evident that the decomposition of hydroxylamine which occurs in solutions not strongly acid is an oxidation, for vigorous boiling of hydroxylamine hydrochloride with $N/10$ NaOH in an inert atmosphere resulted in but slight decomposition.

Losses of hydroxylamine from solution occurred in the presence of nitrite, whatever the reaction of the medium. For example, a solution was prepared containing 0·039 g. NH_2OH-N and 0·005 g. nitrite-N per 100 ml. solution. Immediately after preparation, the hydroxylamine content dropped to 0·036 g. NH_2OH-N per 100 ml. solution, and 23 hours later the solution contained 0·034 g. NH_2OH-N and no nitrite-N per 100 ml. solution. Thus, the whole of the nitrite *plus* a stoichiometrically equivalent amount of hydroxylamine had disappeared within a day.

In a further experiment, a solution containing initially 0·030 g. NH_2OH-N and 0·057 g. nitrite-N per 100 ml. solution had the composition 0 g. NH_2OH-N and 0·043 g. nitrite-N per 100 ml. solution on the following day. Both experiments were carried out at 32°.

It is evident that the course of the reaction between hydroxylamine and nitrous acid must be affected by the reaction of the medium, and accordingly experiments were carried out, in stoppered flasks, at different p_H values (Table VI).

Table VI. *Loss of nitrogen from solutions of hydroxylamine and nitrous acid at different* p_H *values.*

Initial conc. 0·036 g. NH_2OH-N and 0·034 g. nitrite-N per 100 ml. solution. Temperature 32°.

p_H of solution	NH_2OH-N after 1 day	Nitrite-N after 1 day
2·4	0·001	0·000
4·8	0·000	0·000
5·9	0·000	0·016
7·6	0·000	0·028

The photochemical decomposition of ammonium nitrite.

Irradiation of an aqueous solution of ammonium nitrite by means of a 100 D.C. mercury arc gave the results shown in Table VII.

Table VII. *Irradiation of ammonium nitrite by ultraviolet light.*

Initial conc. 0·028 mg. NH_3-N and 0·027 mg. nitrite-N per 100 ml. solution (prepared from ammonium chloride and sodium nitrite).

Treatment	NH_3-N	Nitrite-N
3 months in dark	0·027	0·028
87 hours' irradiation by mercury arc	0·023	0·028

The biological oxidation of ammonium sulphate. Beesley's medium.

The medium employed for the culture of the nitrifying organisms was that recommended by Beesley [1914] and prepared according to the formula:

Nitrogen (in combination as ammonium sulphate) 0·20 g.; KH_2PO_4 0·20 g.; NaCl 0·20 g.; $MgSO_4, 7H_2O$ 0·066 g.; $CaCO_3$ 20·0 g.; distilled water 1000 ml.

The p_H of the medium was 6·9 (quinhydrone electrode) 1½ hours after preparation.

Usually about 2 litres of Beesley's medium were prepared and inoculated with about 1 ml. of a vigorous culture of nitrifying organisms obtained from soil: after standing overnight, quantities of 100 or 200 ml. were placed in a number of 500 ml. conical flasks which were closed with rubber bungs or cotton-wool: when rubber bungs were employed, an adequate supply of air was present for complete nitrification. The flasks were incubated at 32° and the contents analysed at convenient intervals.

Analysis of the nitrifying solutions.

Routine analysis of Beesley's medium, after inoculation with nitrifying organisms, was carried out as follows. The calcium carbonate was removed by filtration and the filtrate made up to a standard volume with water. After qualitative tests for hydroxylamine (Fehling's solution), hyponitrous acid (resorcinol and potassium periodate), nitrous acid (Griess) and nitric acid (reduction to nitrite), aliquot portions of the liquid were analysed as follows.

(*a*) Ammonia was estimated by distillation with magnesia into standard sulphuric acid solution.

(*b*) Nitrite and nitrate together were determined by addition of Devarda's alloy to the solution, after the ammonia estimation had been completed, followed by distillation of the ammonia formed by reduction into standard acid as before.

(*c*) Nitrite was determined by the Griess-Ilosva colorimetric method.

(*d*) The solution was titrated with potassium permanganate in presence of sulphuric acid before and after boiling: in this way the nitrite *plus* hyponitrite, and nitrite alone were estimated, but see the remarks on p. 1095. Permanganate titrations were carried out only in the first series of experiments, a gasometric method being subsequently employed for the estimation of hyponitrite.

(*e*) When present in sufficient quantity, hydroxylamine was estimated by boiling with ferric alum and sulphuric acid, followed by titration with potassium permanganate.

(*f*) *The estimation of hyponitrous acid.* The method of estimating hyponitrous acid entailed collection and examination of the gases evolved on boiling. 100 ml. of the solution were placed in a Claisen distillation flask, and after displacement of the air by a current of pure carbon dioxide from a Farmer's apparatus [1920], the contents of the flask were maintained at boiling-point for about 1 hour. The liberated gases (nitrous and nitric oxides and nitrogen) were swept into a Schiff's nitrometer, over strong potassium hydroxide (saturated with nitrous and nitric oxides), by a further current of carbon dioxide. Nitric oxide was estimated by measurement of the diminution in volume after shaking with ferrous sulphate, and nitrous oxide by determination of the volume decrease after sparking in presence of excess of hydrogen. As air was often present, particularly during the initial stages of nitrification experiments, the amount of hydrogen used was found by noting the diminution in volume after further sparking with oxygen. Attempts to measure the proportions of the three gases present by measuring the volume changes, first after sparking with hydrogen and then after exploding with oxygen, often gave results explicable only on the supposition that any nitric oxide present had been largely reduced to ammonia. Nevertheless, the absorption of nitric oxide by ferrous sulphate solution is not an entirely satisfactory procedure, for the reaction is somewhat slow, and it is necessary first to saturate the absorbent with nitrous oxide; moreover, the precipitate formed as a result of potash from the nitrometer coming into contact with ferrous sulphate is often troublesome in the gas burette.

In some experiments, the hyponitrite in the culture solution was actually separated as the silver salt by precipitation with excess of silver nitrate; after standing overnight, the precipitate was washed free from silver nitrate and decomposed by boiling with sulphuric acid. Analysis of the mixed nitrogen gases evolved was carried out in the manner described. Vigorous boiling of a solution of ammonium nitrite with a small amount of calcium carbonate, the first-named being present in the maximum concentration in which it could occur in the culture liquid, resulted in the liberation of so small a quantity of gas that it was evident that no appreciable error was introduced into the hyponitrite estimation as a result of nitrogen gas liberated by the decomposition of any ammonium nitrite present.

The estimation of hyponitrous acid is not easy, and to this fact, doubtless, must be attributed the former lack of convincing evidence that this acid constitutes an intermediate in the oxidation of ammonia to nitrous acid.

Experiments were first conducted with silver hyponitrite, which was prepared as a pale greenish yellow, flocculent precipitate by addition of excess of silver nitrate to a solution of sodium hyponitrite. The salt dried to a green powder, which rapidly darkened when exposed to light. The silver was estimated as chloride and the hyponitrite determined by oxidation of the salt by $N/10$ $KMnO_4$, acidified with sulphuric acid, and collection of the nitrogen gases evolved on heating in a Schiff's nitrometer over potash, as described above. In this procedure a portion of the nitrogen present in the hyponitrite remained in the flask as nitrate (see equations on p. 1096), and was subsequently estimated in the form of ammonia, after reduction with Devarda's alloy. The nitrogenous gases collected in the nitrometer were analysed in the manner already described.

Analysis of the silver salt gave the following results (%):

	Found				Calculated for $Ag_2N_2O_2$
	Preparation (*a*)		Preparation (*b*)		
Ag		75·7 %		74·8	78·25
N as NO	1·51		1·09		
N as N_2O	6·40		8·79		
N as N_2	0·00				
N as NO_3'	2·55		0·08		
*N_2O_2''		22·4		21·6	21·75
		98·1		96·4	100·00

Calcium hyponitrite was prepared by addition of excess of calcium nitrate to a solution of sodium hyponitrite: the resulting precipitate was washed with water, then with alcohol and finally with ether and dried on filter-paper.

Analysis of calcium hyponitrite:

	Found		Calculated for $CaN_2O_2,4H_2O$
Ca		23·35 (23·34) %	23·29
N as NO	1·85		
N as N_2O	9·57		
N as N_2	0·00		
N as NO_3'	4·17		
Total N	15·59		
*N_2O_2''		32·76	34·80
CO_3''		1·43	—
N_2O (by difference)		42·47	41·86

On heating to constant weight at 105°, the loss in weight was 35·41 %.

* This figure was arrived at by addition of the nitrogen found as NO, N_2O, N_2 and NO_3'' and multiplication by the appropriate factor.

In routine analysis of soil, ammonia is estimated by distillation with magnesia and collection in standard acid, whilst nitrite and nitrate together are determined by measurement of the further ammonia liberated by distillation with magnesia after reduction by Devarda's alloy. With pure hydroxylamine hydrochloride practically no "ammonia"-nitrogen is found by distillation with magnesia, but the value for the "nitrate"-nitrogen is between 70 and 90 % of the hydroxylamine-nitrogen known to be present. Hyponitrite is not detected during such soil analysis, since the nitrogen is lost in gaseous form on warming.

Fortunately, hydroxylamine was never present in more than very small amount during the present investigation, so that no error was introduced from this source. Hyponitrite and nitrite do not react but aqueous solutions of calcium hyponitrite slowly decompose on standing. In presence of hyponitrite, nitrite can be accurately estimated by permanganate titration, provided that the hyponitrite is first decomposed by boiling the solution for 10 minutes.

The action of potassium permanganate on hyponitrites. The action of permanganate on hyponitrites was investigated, but the results obtained are somewhat inconclusive.

Kirschner [1898] stated that oxidation of hyponitrous acid by potassium permanganate entailed the consumption of only one atom of oxygen per mol. of acid, but subsequent investigators have disagreed with this view. The present research showed that considerable variation occurs in the ratio $O/H_2N_2O_2$ during the oxidation of hyponitrites by addition of the latter to a cold, acidified solution of potassium permanganate. In general, the equation

$$4H_2N_2O_2 + 4O = 2HNO_3 + 3H_2O + 3N_2O$$

is satisfied, giving a value of unity to the ratio $O/H_2N_2O_2$, as claimed by Kirschner.

Eighteen determinations of the hyponitrite in silver hyponitrite by this method gave a mean value of 20·94 ± 1·31 % as against the theoretical value of 21·75 for pure $Ag_2N_2O_2$, but the figures varied between 11·02 and 31·94 %.

It appears that the equation given above may represent the sum of two reactions:

$$(a)\quad H_2N_2O_2 = H_2O + N_2O,$$

$$(b)\quad H_2N_2O_2 + 4O = 2HNO_3.$$

Under laboratory conditions with cold permanganate and sulphuric acid, the reaction shows a definite tendency to proceed so that three-fourths of the hyponitrous acid decomposes according to (*a*) and one-fourth is oxidised according to (*b*), although this relationship appears to be fortuitous.

SUMMARY.

The oxidation of ammonia (as ammonium sulphate) by cultures of soil micro-organisms proceeds in a variety of ways. The type most frequently encountered was that in which oxidation ceased after about 5 % of the ammonium salt had been converted into nitrite: with other cultures nitrite formation was vigorous and rapid.

The question of the presence of intermediate compounds during the course of nitrification has been investigated in detail and it has been established that, while hydroxylamine has only an ephemeral existence at p_H values of 6 and above, calcium hyponitrite constitutes an important intermediate compound during nitrification by certain strains of soil bacteria. The presence of hyponitrite was detected by the colour given with resorcinol and potassium periodate, and its estimation was effected by collection and examination of the nitrogenous gases liberated by boiling the culture liquids, or by separation of the silver salt, followed by measurement of the nitrogenous gases evolved by heating with sulphuric acid.

In one series of nitrification experiments, ammonia was oxidised to hyponitrite and this was followed by loss of nitrogen: in this particular series neither nitrite nor nitrate was detected at any time. In all cases, subcultures gave precisely similar results.

Nitrification in soils, by biological and chemical agencies, is discussed in the light of the results obtained during the present investigation.

The author wishes to express his indebtedness to the Research Council of Messrs Imperial Chemical Industries, Ltd. for a grant which rendered this investigation possible, and to Mr H. J. Page and Prof. H. Raistrick for their continued interest.

REFERENCES.

Beesley (1914). *J. Chem. Soc.* **105**, 1014.
Corbet (1934). *Biochem. J.* **28**, 1575.
Dhar (1934). *Nature*, **134**, 572.
Farmer (1920). *J. Chem. Soc.* **117**, 1446.
Fraps and Sterges (1935). *Soil Sci.* **39**, 85.
Gopala Rao (1934). *Soil Sci.* **38**, 143.
—— and Dhar (1931). *Soil Sci.* **31**, 379.
Hoerlin (1934). *Physikal. Z.* **35**, 793.
Jenny (1929). *Soil Sci.* **27**, 169.
Kirschner (1898). *Z. anorg. Chem.* **16**, 424.
Weith and Weber (1874). *Ber. deutsch. chem. Ges.* **7**, 1745.

Note added April 26th, 1935. It has been found that the losses of nitrogen in gaseous form, which occurred in the fourth series of experiments, are attributable, at least in part. to the release of nitrous oxide. Berthelot and Gaudechon [*Compt. Rend. Acad. Sci.* (1910) **150**, 1517] found that nitrous oxide was decomposed into its elements under the action of ultraviolet light and this would explain why no accumulation of this gas takes places in the atmosphere.

CXXXVIII. STUDIES IN TISSUE METABOLISM.
VI. LACTIC DEHYDROGENASE, XANTHINE OXIDASE AND NUCLEOSIDASE IN TUMOUR AND MUSCLE EXTRACTS.

By ERIC BOYLAND AND MARGARET ESTHER BOYLAND.

From the Research Institute, The Cancer Hospital, London, S.W. 3.

(*Received March 29th, 1935.*)

In a previous communication [Boyland and Boyland, 1934] two distinct lactic dehydrogenases were described, one present in yeast and one in mammalian muscle. Tumour tissue (Jensen rat sarcoma) extracts made by the method previously used with muscle reduced methylene blue rapidly in the presence of lactic dehydrogenase coenzyme (prepared after Banga *et al.* [1932]) in the absence of any other substrate. Extracts of J.R.S. were also made by the method of freezing and thawing described by Boyland and Mawson [1934] with similar results. No amount of washing of the tissue or dialysis of the extract appeared to reduce the activity. In some cases the time of reduction of methylene blue was slightly decreased by the addition of lactate but the differences appeared to bear no relation to the time of dialysis, which precluded the possibility that the extract was acting on some dialysable substrate which it contained. Similarly extracts of rat and mouse muscle contained some lactic dehydrogenase but also reduced methylene blue without the addition of lactate. Some typical results for muscle extracts are given in Table I. All the results described in this paper were obtained by observation of the time necessary to decolorise methylene blue solution in evacuated Thunberg tubes at 38°.

Table I. *Reduction of methylene blue by frozen muscle extracts.*

1 ml. extract, 0·5 ml. $M/15$ Na_2HPO_4, 0·5 ml. substrate + water, 0·5 ml. 1/5000 methylene blue p_H 8·0–8·2.

Muscle	Rat skeletal		Rat heart		Mouse skeletal		Rabbit heart		Rabbit skeletal	
Additions	T mins.	Activity (100/T)	T mins.	Activity (100/T)	T mins.	Activity (100/T)	T mins.	Activity (100/T)	T mins.	Activity (100/T)
None	>180	0·0	>800	0·0	>600	0·0	>180	0·0	>180	0·0
Lactate ($M/50$)	>180	0·0	—	—	>600	0·0	>180	0·0	>180	0·0
Coenzyme (0·4 mg.)	29	3·4	24	4·2	70	1·7	>180	0·0	>180	0·0
Coenzyme + lactate	18	5·5	1½	66·0	36	2·8	17	5·9	16	6·2
Adenyl pyrophosphate ($M/1000$)	22	4·3	16	6·2	90	1·1	>180	0·0	>180	0·0
Hypoxanthine ($M/6000$)	19	5·2	16	6·2	35	2·9	>180	0·0	>180	0·0

It can be seen that extracts of rabbit heart or skeletal muscle will not decolor methylene blue in the presence of coenzyme without added lactate.

Extracts of some tumours (carcinoma 63, sarcoma 37, Crocker 180 and Mal sarcoma of mice and rat sarcoma R. 10) gave results comparable with those obtained with muscle, *i.e.* a lactic dehydrogenase was present but its effect was partly masked by the presence of another enzyme system. It will be shown that this could be adenine nucleosidase and xanthine oxidase. The optimum reaction for the lactic dehydrogenase of heart muscle is at about p_H 9, while at p_H 7 the enzyme has about half the optimum activity [Boyland and Boyland, 1934]. Experiments showing the effect of p_H on a tumour extract are given in Table II and Fig. 1.

Table II. *Reduction of methylene blue by extracts of frozen J.R.S.*

	p_H	Coenzyme mg.	Lactate (M/50)	T mins.	Activity (100/T)
Extract 1	6·0	0·4	–	5	20·0
	6·0	0·4	+	8½	12·0
	8·5	0·4	–	7	14·0
	8·5	0·4	+	5½	18·0
Extract 2	8·0	0·4	–	9	11·0
	8·0	0·4	+	8	12·0
	8·0	0·1	–	90	1·1
	8·0	0·1	+	34	2·9

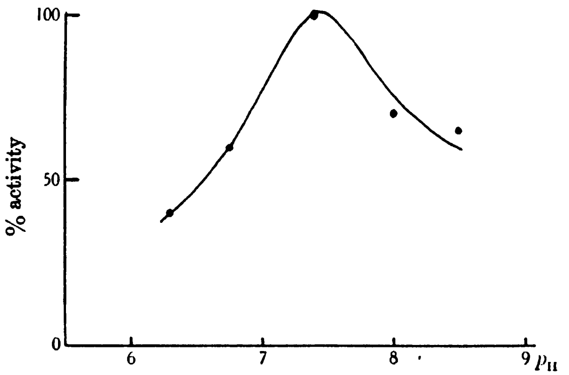

Fig. 1. Reduction of methylene blue by extract of frozen J.R.S. In each tube 1 ml. extract, 1 ml. M/15 phosphate, adenylpyrophosphate (M/1000), and 1 ml. M/5000 methylene blue.

Table II also shows the effect of adding small quantities of coenzyme, sufficient to activate the lactic dehydrogenase but barely enough to act as substrate for xanthine dehydrogenase, thus revealing the effect of lactic dehydrogenase. With sarcomata 37, Crocker 180 and Mal[1] the proportion of the lactic dehydrogenase to the nucleosidase-xanthine oxidase systems is much higher as shown in Table III.

The rate of oxidation of hexosediphosphate in the presence of coenzyme was determined in some extracts and was found to be similar to that of lactic acid in all experiments. It is therefore not tabulated in Table III.

Xanthine oxidase.

The addition of hypoxanthine solution (final concentration 1 in 2000) to the tissue extracts produced decoloration of methylene blue in all cases except those of rabbit skeletal and heart muscle. Figures for xanthine oxidase activity are given in Tables I and III under hypoxanthine.

A measure of the relative amounts of the various enzyme systems is given by the figures in Tables I and III for the "activity" (100/T) with the corresponding substances. The figure for lactic dehydrogenase (column headed lactate

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[1] A tumour produced in mice by Mr H. Burrows by subcutaneous injection of sodium 1:2:5:6-dibenzanthracene-9:10-endo-$\alpha\beta$-succinate [Cook, 1931].

Table III. *Activity of frozen tumour extracts.*

1 ml. extract, 0·5 ml. $M/15$ Na_2HPO_4, substrate and water 0·5 ml., 0·5 ml. 1/5000 methylene blue.

In all cases the reduction times with extract alone, and with lactate+extract were long compared with the other times.

(*a*) and (*b*) refer to extracts of different batches of tumours.

Substrate		Activity 100/T		
		Lactate $M/50$	Adenylpyrophosphate $M/1000$	Hypoxanthine $M/6000$
Tumour				
J.R.S.	(*a*)	0·0	12·5	33·0
	(*b* 1)	4·4	20·0	20·0
	(*b* 2)*	2·2	17·0	18·0
R. 10	(*a*)	0·0†	17·0	18·2
	(*b*)	0·0	9·5	15·4
Crocker 180	(*a*)	3·5	3·2	4·5
	(*b*)	12·0	5·0	8·0
C 63	(*a*)	0·9	2·7	10·0
	(*b*)	0·0	1·4	6·0
S 37	(*a*)	19·0	12·0	17·0
	(*b*)	16·0	12·0	12·0
Mal. sarcoma	(*a*)	3·9	1·0	3·2
	(*b*)	12·2	6·0	6·0

* Second extraction of same tumour. † With half quantity of coenzyme 100/T was 0·4.

$M/50$) in Table III was obtained by subtracting the "activity" with coenzyme alone from that with coenzyme+lactate. Zero activity therefore implies that the reduction times with coenzyme, with and without lactate, were the same. In some cases the times were actually longer with lactate than without. This may be due to a protection of the coenzyme by lactate. The figures for lactic dehydrogenase are therefore minimum values. The same effect is shown with adenylpyrophosphate and lactate or hexosediphosphate in Table IV.

Table IV. *Reduction of methylene blue by extracts of Mal. sarcoma.*

Additions	Extract 1		Extract 2	
	T mins.	Activity (100/T)	T mins.	Activity (100/T)
Adenylpyrophosphate ($M/1000$)	14	7·2	16	6·2
Adenylpyrophosphate+lactate ($M/50$)	19	5·3	39	2·5
Adenylpyrophosphate+hexosediphosphate ($M/50$)	27	3·7	38	2·6
Coenzyme (0·4 mg.)	27	3·7	37	2·7
Coenzyme+lactate	7	14·0	16	6·2
Coenzyme+hexosediphosphate	6½	15·0	16	6·2

Table IV also shows that adenylpyrophosphate has no activity as lactic dehydrogenase coenzyme. This has been confirmed by testing with the lactic dehydrogenase preparation of rabbit muscle, which contains no xanthine oxidase (see Table I) and does not decolorise methylene blue in the presence of adenylpyrophosphate alone.

Nucleosidase.

Szent-Györgyi's coenzyme, adenylpyrophosphate (prepared according to Lohmann [1932]) and adenylic acid reduced methylene blue in the presence of tumour extracts, but not with rabbit muscle extracts. The effect is probably due to the combined actions of a nucleosidase (adenine nucleosidase) and xanthine oxidise. It cannot be due to the latter enzyme acting with an impurity in the preparations, for there is a definite optimum p_H at about 7·4 (see Fig. 1), whereas xanthine oxidase has an almost constant activity between p_H 6·5 and 8·5. Levene *et al.* [1924] have shown that nucleosidase has an optimum at p_H 7·5.

A minimum figure for the nucleosidase activity of the extracts is given by the activity in the presence of adenylpyrophosphate, for the enzyme must act on this before the dehydrogenation can occur. That the limiting factor is often the amount of xanthine oxidase present can be seen from the fact that the reduction times are in many cases nearly the same for hypoxanthine and adenylpyrophosphate. On the other hand milk xanthine oxidase reduces methylene blue 100 times more rapidly in the presence of hypoxanthine than in the presence of adenylic acid [Dixon and Lemberg, 1934]. Rabbit muscle extracts do not reduce methylene blue with adenylpyrophosphate or hypoxanthine. The lack of activity with adenyl pyrophosphate may be due to the absence of xanthine oxidase only and not of nucleosidase.

Incubation of rabbit muscle extract with coenzyme for 50 minutes did not however destroy the latter, for its power of acting as lactic dehydrogenase coenzyme was unimpaired. If adenine nucleosidase is present it must therefore be only in relatively small amounts.

DISCUSSION.

The foregoing experiments show that the tissue of the tumours examined contains lactic dehydrogenase, which requires a coenzyme for its action and shows optimum activity in alkaline solutions. It therefore appears to be identical with the enzyme present in mammalian muscle. Many tumour extracts however oxidise the coenzyme so rapidly that the lactic acid oxidation is not readily observed. Rat and mouse muscle extracts resemble the tumour extracts to some extent in this respect as they reduce methylene blue in the presence of coenzyme or adenylpyrophosphate. In this they differ markedly from rabbit muscle extract which does not oxidise the coenzyme, nor does it contain xanthine oxidase. It is possibly for this reason that Meyerhof found that rat muscle did not give glycolytically active extracts, as his first experiments were made without addition of coenzyme or adenylpyrophosphate. Boyland and Mawson [1934] were able to prepare glycolytically active rat muscle extracts by adding sufficient adenylpyrophosphate to compensate for its destruction.

Tumour extracts generally attack adenylpyrophosphate and lactic dehydrogenase coenzyme even more rapidly than does rat muscle extract. There is a large variation in the rate of attack with different tumour extracts. Extracts of J.R.S. oxidise the coenzyme so rapidly that it is difficult to determine the lactic dehydrogenase activity with any degree of accuracy. On the other hand some extracts of rapidly growing mouse tumours do not react so rapidly with the coenzyme and therefore show lactic dehydrogenase activity.

SUMMARY.

1. Rat, mouse and rabbit muscle and mouse and rat tumours (C 63, S 37, Crocker 180, Mal. sarcoma, R. 10 and J.R.S.) contain lactic dehydrogenase similar to that found in heart muscle.

2. Rabbit muscle extract does not contain xanthine oxidase or adenine nucleosidase but rat and mouse muscle extracts contain both these enzymes.

3. All the rat and mouse tumour extracts examined contain xanthine oxidase and attack adenylpyrophosphate or lactic dehydrogenase coenzyme rapidly. The rate at which the adenine derivatives are attacked varies with different tumour strains.

One of us (M. E. B.) has pleasure in thanking the House Committee of the Cancer Hospital for a scholarship held during the progress of this work.

REFERENCES.

Banga, Szent-Gyorgyi and Vargha (1932). *Z. physiol. Chem.* **210**, 228.
Boyland and Boyland (1934). *Biochem. J.* **28**, 1417.
—— and Mawson (1934). *Biochem. J.* **28**, 1409.
Cook (1931). *J. Chem. Soc.* 3277.
Dixon and Lemberg (1934). *Biochem. J.* **28**, 2065.
Levene, Yamagawa and Weber (1924). *J. Biol. Chem.* **60**, 693.
Lohmann (1932). *Biochem. Z.* **254**, 381.

CXXXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLIV. FULVIC ACID, A NEW CRYSTALLINE YELLOW PIGMENT, A METABOLIC PRODUCT OF *P. GRISEO-FULVUM* DIERCKX, *P. FLEXUOSUM* DALE AND *P. BREFELDIANUM* DODGE.

BY ALBERT EDWARD OXFORD, HAROLD RAISTRICK
AND PAUL SIMONART.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received March 29th, 1935.*)

It has already been reported in Parts XIX and XXIX of this series that when *Penicillium griseo-fulvum* Dierckx is grown on liquid media containing glucose as sole source of carbon, and sodium nitrate as sole source of nitrogen, the following products are to be found in the metabolism solution when most of the glucose has been utilised: 6-methylsalicylic acid [Anslow and Raistrick, 1931]; gentisic acid, fumaric acid and mannitol [Raistrick and Simonart, 1933]. The factors governing the formation of these products are temperature of incubation and ratio of glucose to sodium nitrate. The presence of the two phenolic acids mentioned above causes the metabolism solutions to give respectively a purple or blue coloration with ferric chloride. Further experiments have now shown that when the nitrogen source is changed from NO_3^- to NH_4^+ the course of the metabolism of the glucose is entirely changed. The coloration given with ferric chloride is now no longer purple or blue but green, and in place of the above two colourless phenolic acids there is formed a much more complex yellow phenolic acid, of empirical formula $C_{14}H_{12}O_8$, which gives a deep green colour with aqueous ferric chloride, and for which we propose the name *fulvic acid*. The yield of fulvic acid from this organism is however small and its isolation in a pure state from the metabolism solution is very difficult. The reaction given by fulvic acid with ferric chloride and also certain of its other properties are very similar to those of the complex mould product citromycetin, $C_{14}H_{10}O_7$, produced by species of *Citromyces* [Hetherington and Raistrick, 1931], hitherto the only mould metabolic product known to give an intense green colour with ferric chloride.

Subsequently to the above observations on *P. griseo-fulvum* our colleague, Mr G. Smith, has found that two other species of *Penicillium*, *viz. P. flexuosum* Dale and *P. Brefeldianum* Dodge[1] give rise to metabolic solutions giving intense green colorations with ferric chloride, when grown in test-tubes on Raulin-Thom medium containing glucose and tartaric acid as sources of carbon, and ammonium tartrate, phosphate and sulphate as sources of nitrogen. We have isolated the metabolic product responsible for these reactions and have found that it is in fact fulvic acid in each case. Furthermore, the yield, especially with *P. Brefeldianum*, is very much larger than with *P. griseo-fulvum*, the greater part of the fulvic acid being found however in the mycelium in the case of *P. Brefeldianum*.

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[1] This name has recently been altered to *Carpenteles Brefeldianum* by Shear [1934].

A further point of interest is that the metabolism solution from *P. flexuosum* also contains 6-methylsalicylic acid, but this acid was not detected as a metabolic product of *P. Brefeldianum*. The fact that both *P. griseo-fulvum* and *P. flexuosum* give rise to fulvic and 6-methylsalicylic acids is not surprising in view of the close morphological relationship of these organisms. Thom [1930] places *P. griseo-fulvum* in section 5 of the *Asymmetrica* division of species of *Penicillia*, *viz.* among the *Asymmetrica-funiculosa*, with possible affinities with the next section, *viz.* the *Fasciculata*, in which he places *P. flexuosum*.

The relationship between these organisms and *P. Brefeldianum* appears however to be remote. Dodge [1933] has recently made a careful study of the last-named organism, which was first isolated by Dr Rhoda Benham in America from the human alimentary tract, and reports that in its conidial stage it belongs to the *Monoverticillata* section of *Penicillia* (as do *Citromyces* species) with the further note that in this stage it may possibly belong to Thom's *Asymmetrica* section. It is of great interest that *P. Brefeldianum* readily produces perithecia, quite unlike those of species in the *P. luteum* series but comparable with those described long ago by Brefeld [1874] as typical of the so-called *P. glaucum* Link, an organism which no later worker has been able to isolate and the very existence of which has been doubted by many mycologists. Dodge, in fact, is of the opinion that *P. Brefeldianum* is morphologically very near to the classical *P. glaucum* described by Brefeld. It may be noted here that *P. javanicum* van Beijma [van Beijma, 1929] which is morphologically very close to *P. Brefeldianum*, and which also forms similar perithecia, appears to metabolise glucose in a medium containing NH_4NO_3 as source of nitrogen in an entirely different way [Lockwood *et al.*, 1934], to yield citric acid, a product which was not detected among the metabolic products of *P. Brefeldianum*. We intend at an early date to study in detail the metabolism of *P. javanicum* when grown on Raulin-Thom medium and also the metabolism of both organisms when grown on Czapek-Dox medium with sodium nitrate as sole source of nitrogen.

The mycelium of *P. griseo-fulvum* has been shown to contain a colourless, crystalline, nitrogenous substance (M.P. 165° decomp.) while the mycelium of *P. Brefeldianum* yields, in addition to fulvic acid, a different nitrogenous compound, as colourless crystals, M.P. 132–135°. The description of these two metabolic products is reserved for a later communication.

In this paper we propose to record the isolation, properties, derivatives and reactions of fulvic acid, together with the evidence for the empirical formula we have assigned to it, *viz.* $C_{14}H_{12}O_8$. The question of its structural formula is left for a later communication.

EXPERIMENTAL.

Organisms used.

(*a*) *P. griseo-fulvum* Dierckx. L.S.H.T.M. Catalogue No. P 38. Received from Prof. Ph. Biourge, University of Louvain, December 1929, as No. B 34.

(*b*) *P. flexuosum* Dale. L.S.H.T.M. Catalogue No. P 129. Received from Centraalbureau voor Schimmelcultures, Baarn, August 1931. Supplied by Dale.

(*c*) *P. Brefeldianum* Dodge. L.S.H.T.M. Catalogue No. P 157. Received from Baarn, September 1933. Supplied by Dodge.

Section A. Metabolic products of P. griseo-fulvum *grown on media containing glucose and tartaric acid as sources of carbon, and ammonium salts as sources of nitrogen*[1].

The standard media used in this laboratory are Czapek-Dox medium (glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; distilled water, 1 litre), and Raulin-Thom medium (glucose, 50 g.; tartaric acid, 2·7 g.; ammonium tartrate, 2·7 g.; diammonium hydrogen phosphate, 0·4 g.; K_2CO_3, 0·4 g.; $MgCO_3$, 0·27 g.; $(NH_4)_2SO_4$, 0·17 g.; $ZnSO_4$, $7H_2O$, 0·05 g.; $FeSO_4$, $7H_2O$, 0·05 g.; distilled water, 1 litre).

Preliminary experiments in test-tubes indicated that when the mould was grown on Raulin-Thom medium, the metabolism solution, after a few days' incubation, gave a green colour with ferric chloride. The optimum temperature for the production of the substance responsible for the coloration appeared to be about 20°, but there still remained 3·28 % of glucose after 42 days' incubation. The medium, initially at p_H 4·1, became much more strongly acid during the incubation period, *e.g.*, in the instance quoted above the p_H was below 2·8 after 42 days' incubation. On the other hand, when the mould was grown on Czapek-Dox medium at the same temperature the metabolism solution was slightly alkaline when all the glucose had been utilised, and gave a purple colour with $FeCl_3$. Hence it obviously became desirable to find out which constituents of Raulin-Thom medium are essential for the formation of the product giving the green coloration with $FeCl_3$.

A comparison of the respective compositions of Raulin-Thom and Czapek-Dox media shows that the former contains NH_4^+, CO_3^{--}, Zn^{++} and tartaric acid, all absent from the latter, whilst the latter contains Na^+, Cl^- and NO_3^- all absent from the former. Experiments were therefore conducted with the following four media:

A. Czapek-Dox medium omitting KCl but with $ZnSO_4$ added.
B. Czapek-Dox medium omitting $NaNO_3$ but with NH_4NO_3 added.
C. Czapek-Dox medium omitting $NaNO_3$ but with ammonium tartrate added.
D. Czapek-Dox medium with K_2CO_3 added.

After 14 days' incubation at 20°, only the metabolism solutions from B and C gave the green ferric chloride reaction. It appeared therefore that replacement of NO_3^- by NH_4^+ was responsible for the new mode of metabolism (tartrate not being essential), and different sources of NH_4^+ were next tried, *viz.* ammonium tartrate, sulphate and phosphate at concentrations of 1, 2, 3, 4 and 5 g. per litre. The results indicated that ammonium tartrate at a concentration of 5 g. per litre was the best source of NH_4^+. Finally a number of media were made up containing the salts of Czapek-Dox medium but with ammonium tartrate (5 g. per litre) replacing sodium nitrate, and with varying concentrations of glucose up to 10 %. The tubes were incubated for 30 days at 21°. The results are summarised in Table I and show that good production of phenolic substances took place on media containing 3–10 % glucose. In all cases a dark green colour was obtained with $FeCl_3$.

Isolation of the metabolic product responsible for the green coloration with ferric chloride. The medium selected for large scale work had the following composition: glucose, 50 g.; ammonium tartrate, 5 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$,

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[1] The results in this section are taken from a thesis presented in 1934 by Dr P. Simonart for the degree of Ph.D. in the University of London.

Table I.

% glucose at start	Apparent % glucose by polarimeter at end	Br absorption value at end. mg. per ml.	Final p_H
1	—	0·93	7·1
2	0·21	2·05	4·1
3	0·64	2·53	3·5
4	1·70	2·40	3·2
5	2·70	2·29	3·2
6	3·66	2·46	3·1
7	4·80	2·44	3·2
8	5·88	2·48	3·2
9	7·06	2·43	3·2
10	7·80	2·56	3·3

$7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1 litre. One hundred and fifty 1 litre flasks each containing 350 ml. of this medium were sterilised by steaming on each of three successive days, sown with a suspension of spores of *P. griseo-fulvum* (obtained from 15-day old cultures at 30° on beer-wort agar in Roux bottles) and incubated at room temperature (19–21°) for 50 days. The mycelium and the filtered metabolism solution, which gave an intense green colour with $FeCl_3$, were then dealt with separately. The mycelial constituents of *P. griseo-fulvum* grown on Czapek-Dox medium and on the above modification of this medium will form the subject of a separate paper. In this place it will be sufficient to say that both mycelia contained considerable amounts of an ether-soluble, colourless, crystalline nitrogenous substance (M.P. 165° decomp.) the isolation of which in a crude state (M.P. about 150°) was mentioned by Anslow and Raistrick [1931] in their first biochemical study of this mould; and that the mycelium from the modified Czapek-Dox medium appeared not to contain any of the phenolic substance (fulvic acid) present in the metabolism solution.

Estimations carried out on an average sample of the metabolism solution gave the following results: apparent glucose content (by polarimeter) 1·82 %; Br absorption value (Koppeschaar's method) 2·1 mg. per ml. The metabolism solution was evaporated to small volume *in vacuo* below 50°, made strongly acid to Congo red by addition of 2*N* H_2SO_4 and extracted 12 times with an equal volume of chloroform. The extract was dried over anhydrous sodium sulphate and the solvent removed *in vacuo* at 40°. The brown, gummy residue was treated with a large volume of boiling water, the aqueous extract boiled with a little decolorising charcoal and filtered. On cooling, 0·42 g. of yellow needles separated, M.P. 241° decomp. The substance was further purified by crystallisation from ethyl acetate. Analysis and mixed M.P. with specimens of fulvic acid produced by *P. flexuosum* and *P. Brefeldianum* will be found later (p. 1111).

Section B. Metabolic products of P. flexuosum *Dale grown on Raulin-Thom medium.*

A preliminary investigation of the course of metabolism of glucose by this mould was made exactly as described in Part XXXIX of this series [Oxford and Raistrick, 1934] for *P. crateriforme* Gilman and Abbott. A quantity of Raulin-Thom medium (for composition see p. 1104) was distributed in 350 ml. lots in 49 one-litre conical flasks. After sterilisation each flask was sown with a spore suspension of *P. flexuosum* and incubated at 24°. Single flasks were removed periodically and tests made on the filtered metabolism solution. The quantitative results so obtained are summarised in Table II.

Table II.

Period of incubation days	Apparent % glucose by polarimeter	% glucose by Shaffer-Hartmann method	p_H	Titratable acidity ml. $N/10$ per 10 ml.	Br absorption value mg./ml.
0	5·35	5·20	3·9	2·5	0·10
3 (complete mycelial felt)	4·70	—	3·5	3·7	0·30
6	2·76	2·70	2·8	4·6	0·49
9	1·23	1·21	3·0	2·6	0·68
13	0·50	0·50	3·6	0·8	0·88
16	0·25	—	4·2	—	1·00
22	Practically nil	Present	5·6	—	1·20
27	Nil	Nil	6·3	—	1·22

The only qualitative results of note were the following: the green coloration with ferric chloride was perceptible on the third day, of moderate intensity on the ninth day and a deep emerald green on the thirteenth and subsequent days. Tartrate was still present on the ninth day but absent later. A moderate yellow precipitate was given with normal lead acetate on the 13th and subsequent days. The metabolism solution in the later stages was pale yellowish brown, deepening to reddish brown in colour on the twenty-second day; when brought to p_H 7 a deeper orange-brown or red colour was obtained. The mycelial felt, coherent at first, began to disintegrate about the twenty-second day and marked autolysis had set in by the twenty-seventh day. It is evident that the mould grows, and metabolises glucose, rapidly, but the yield of dry mycelium (5·1 g. per flask) was not excessively large. Solvent extraction of this mycelium yielded a minute amount of fulvic acid, but none of the nitrogenous substance, M.P. 165°, found in the mycelium of *P. griseo-fulvum*.

Isolation of fulvic acid from the metabolism solution. 44 flasks were removed after 16 days' incubation and the filtered metabolism solution was evaporated to 400 ml. *in vacuo* below 40°. A dark-coloured precipitate (A) separated during the evaporation (4·6 g.), which was found to contain the greater part of the fulvic acid produced by the mould. A minute amount of fulvic acid (M.P. 236° decomp.) was with difficulty obtained from the filtrate from this precipitate by a method similar to that previously described for *P. griseo-fulvum*, or by addition of normal lead acetate, treatment of the lead precipitate with dilute H_2SO_4, extraction with ether and crystallisation from water.

The dry precipitate (A) was extracted with ether in a Soxhlet apparatus for 3 working days. About 1·5 g. of yellow material separated from the ethereal extract and was crystallised first from 20 ml. dioxan, then from 16 ml. dioxan. Yield: 0·4 g. of lemon-yellow rods; M.P. 242° decomp. The material in the thimble unextracted by ether still contained some fulvic acid, as was proved by extraction with boiling dioxan. No other crystalline substance was obtained from it.

Isolation of 6-methylsalicylic acid from the metabolism solution. The dark brown filtrate from precipitate (A) was made acid to Congo red by addition of $2N$ H_2SO_4 and extracted 3 times with an equal volume of ether. Evaporation of the dried ethereal extract yielded a brown gum (1·4 g.) which, by fractional crystallisation from water, was shown to contain a little fulvic acid together with small amounts of 6-methylsalicylic acid. This substance was obtained as colourless crystals which were purified by sublimation in a high vacuum to yield colourless prisms, M.P. 170–171°, alone or mixed with authentic 6-methylsalicylic acid, which gave a beautiful purple colour with aqueous ferric chloride.

Section C. Metabolic products of P. Brefeldianum *Dodge grown on Raulin-Thom medium.*

A preliminary survey carried out as outlined previously for *P. flexuosum* Dale indicated that the course of metabolism was very similar to that of the latter mould. Growth was fairly rapid with production of a thick tough mycelial felt which, after becoming depressed at the edges, eventually became waterlogged, sank and was replaced by a new similar surface felt. The turbid metabolism solution was brown in colour and was obtained clear only after repeated filtration through paper. Its p_H was always well on the acid side (4 or below) and the titratable acidity (ml. $N/10$ NaOH required to bring 10 ml. of metabolism solution to p_H 7) initially 2·5, rose to 5·8 on the sixth day and then fell to 1·0 on the twenty-seventh day when all the glucose had been metabolised. When brought to p_H 7 the metabolism solution became deep brownish red in colour (or even clear wine-red when made slightly alkaline), but the colour became lighter on making strongly alkaline. The bromine absorption value of the metabolism solution steadily increased with incubation and when all the glucose had been utilised was 2·40 mg./ml. At all times the glucose content as estimated polarimetrically was practically identical with the value obtained by the Shaffer-Hartmann method. At the end of the incubation period the only striking reactions given by the metabolism solution were the following: an intense emerald-green coloration with aqueous or aqueous-alcoholic ferric chloride, given definitely on the sixth day and later becoming more striking; a heavy, brown precipitate with mercuric chloride solution; a heavy, yellow precipitate with normal lead acetate; reduction of $AgNO_3$ on standing. At p_H 7 the ferric chloride coloration is brown, becoming green on addition of a drop or two of dilute HCl.

Since the yield of fulvic acid given by *P. Brefeldianum* is considerably larger than with *P. griseo-fulvum* or *P. flexuosum*, *P. Brefeldianum* was used for the preparation in quantity of fulvic acid. The following are the results for a typical large-scale experiment: 100 flasks, each containing 350 ml. of medium, were taken after 33 days' incubation (residual glucose, nil) and the mycelium was separated from the bulk of the metabolism solution by filtration through muslin (weight of dried mycelium, 496 g.). The turbid filtrate was filtered through paper and the dark brown, slimy precipitate (product A, 15 g.) collected. The final clear brown filtrate was evaporated to about 400 ml. *in vacuo* below 50° and left in the cold room for several days. The brown, partly crystalline precipitate which separated was filtered off, washed with water and dried (product B, 42 g.). Further acidification of the final filtrate threw down a little tarry material from which no crystalline product could be isolated. Repeated ethereal extraction of the acid filtrate from this tar yielded a brown gum from which a minute amount of fulvic acid was eventually isolated by repeated crystallisation from water (charcoal) and finally from aqueous dioxan. The various aqueous mother liquors from these crystallisations were evaporated down in stages, but no product was obtained which gave other than a green coloration with ferric chloride. Hence 6-methylsalicylic acid was almost certainly absent.

Further batches of 100 flasks each gave very similar results save that the weight of dry mycelium fluctuated somewhat, *e.g.* weights of mycelium from successive batches were 452; 496; 371; 184; 351 g.; the probable explanation being that the last 3 batches were given longer periods of incubation (40–50 days), *i.e.* until several days after all the glucose had disappeared, with resulting partial autolysis of the mycelial felt. The yield of fulvic acid appeared not to be adversely affected thereby, but in these instances product B was small, its counterpart being the precipitate obtained by acidification of the mother liquor from product B (previously a tar).

Isolation and separation of fulvic acid from other metabolic products. The isolation of fulvic acid in quantity and in a high state of purity proved to be difficult for many reasons:

(*a*) Fulvic acid is only very sparingly soluble in hot ether or chloroform. To extract all the fulvic acid from the mycelium, or from product B, by use of these solvents in a Soxhlet apparatus requires weeks or even months.

(*b*) The crude fulvic acid so obtained cannot be satisfactorily purified from solvents such as hot ethyl acetate or dioxan, in which it is moderately soluble, because the accompanying nitrogenous substance, M.P. 132–135°, is very similar to fulvic acid in solubilities and crystalline shape.

(*c*) Even when the fulvic acid has been separated from this nitrogenous substance and other neutral substances by solution in aqueous sodium bicarbonate, the product recovered on acidification contains other phenolic acids, which are more sparingly soluble in dioxan than is fulvic acid and cannot be removed with certainty by crystallisation from that solvent. It is necessary first to extract the fulvic acid with boiling ethyl acetate in which the other phenolic acids are practically insoluble.

(*d*) The most efficient method of purification, *viz.* conversion into the potassium salt and recrystallisation of the latter, is impracticable on a large scale since the solubility of this salt in boiling methyl alcohol is only of the order of 0·1 %.

The following paragraphs give an account of the method finally adopted for the separation of the metabolic products and the purification of the crude fulvic acid. The mycelium and products A and B were worked up separately.

Treatment of the mycelium. The dried mycelium was finely powdered and extracted with light petroleum (B.P. 50–60°) in a large Soxhlet apparatus for two working days. This treatment removes fats and sterols, in which the mycelium is particularly rich. Thus, if the extract is evaporated to small bulk and allowed to stand for several weeks in the cold room, a copious crystalline precipitate of ergosterol (long needles) and, probably, ergosteryl esters (groups of small crystals) is produced; or if the extract is evaporated to dryness and treated with cold alcohol [Oxford and Raistrick, 1933] a relatively large amount of insoluble ergosteryl esters is obtained. The defatted mycelium was then similarly extracted with ether until it appeared to be entirely exhausted of ether-soluble material. This process required at least 40–50 working days and the mycelium was reground and redistributed among the thimbles after about 20 days' extraction. The yellowish brown solid crust which separated from the ether on the walls of the flasks was removed periodically, and the solvent itself was likewise renewed every 5 or 6 days, the ethereal mother liquors being kept and later combined, evaporated to dryness and worked up separately. The material which separated from the ether during the first 2 or 3 days' extraction was worked up separately, since it contained the bulk of a new, colourless, crystalline compound containing nitrogen, M.P. 132–135°.

Purification of crude fulvic acid. The crude fulvic acid so obtained (15–20 % by weight of the mycelium) was finely powdered and treated (in 50 g. lots in a large filtrate jar) with stirring, with excess of cold, nearly saturated sodium bicarbonate solution. After the effervescence had subsided, the mixture was allowed to stand for several hours, filtered and the dark-coloured filtrate acidified with $2N$ HCl and then kept in the cold room overnight. The impure fulvic acid, now free from neutral substances, was collected, well washed with water and dried *in vacuo* over solid KOH. It was further worked up in 40 g. lots, each being extracted many times with about 1600 ml. boiling ethyl acetate until the last extract deposited no crystalline material, or a mere trace of a sandy powder on long standing. Four or five extractions were usually necessary. The first was always deep red in colour, and subsequent extracts were orange-red, orange and finally pale yellow or brown. Fulvic acid was deposited in a hard cake of large brown or brownish yellow prisms on the bottoms and walls of the flasks, and since crystallisation was slow, 4 or 5 days were allowed before proceeding to the next stage. Detachment of the deposit by scraping with a spatula is a laborious task and it is better and quicker to decant off the supernatant liquid, wash once or twice with a little ethyl acetate, and dry *in situ* in a stream of air. Tared one-litre conical flasks were used for these crystallisations. Second and third crops of more impure fulvic acid were obtained by evaporating the ethyl acetate mother-liquors in stages and were separately crystallised from dioxan.

The volume of dioxan per g. required for the final crystallisation depends on the purity of the fulvic acid at this stage. If the acid is moderately pure, 10–12 ml. per g. will be required, but if still highly coloured and impure, 6 ml. per g. will be sufficient to bring the material into solution. The mixture of dioxan and crystals was heated to boiling with constant stirring in an oil-bath, boiled for a few minutes and filtered immediately. To the filtrate was added about 10 % of its volume of water and the whole, when cold, was left in the cold room for at least 2 days. Fulvic acid then separated as a yellow, sandy, crystalline powder (very small rods) which showed no tendency to adhere to the walls of the flask. The precipitate was collected, washed with dioxan containing 10 % of water, then with ethyl acetate and dried in air. Second, third, fourth and final crops were obtained by diluting successive mother liquors each time with 20 % of water and allowing to stand for several days in the cold room. The melting point and colour of each crop were noted. In general the lighter the colour, the higher the decomposition point when heated in a M.P. tube at a standard rate. The limiting values of the decomposition point were taken as 235° and 248°. Material melting or decomposing below 235° was again recrystallised from dioxan.

Treatment of product A. Product A was treated in a similar manner. In one instance, the proportion of the crystalline nitrogenous substance, M.P. 132–135°, was exceptionally large. The material remaining unextracted by ether after some 3 weeks' extraction was triturated with sodium bicarbonate solution. The filtrate contained some fulvic acid which was purified as previously described.

Treatment of product B. Product B yielded, by long-continued ethereal extraction, a pale, yellowish brown solid which was almost completely soluble in sodium bicarbonate solution. The unextracted part (after some weeks' extraction) was re-ground and the extraction continued with chloroform. After a few days, nothing further appeared to be extracted by this solvent, but this was due, not to removal of all the fulvic acid, but to the caking together of the solid in the thimble. The extraction was therefore discontinued and the residual solid treated with sodium bicarbonate *etc.*, as previously described. The presence of potassium hydrogen tartrate was noted in this residual solid, and a colourless specimen of this salt was obtained by repeated crystallisation from boiling water.

Recovery of dioxan. All dioxan used in the large scale work described above was purified by the method of Eigenberger [1931; see also Oxford, 1934] and was free from aldehydes and acetals. The final aqueous dioxan mother-liquors were combined and the greater part of the solvent was distilled off. The distillate was treated as crude, technical dioxan and submitted to the full process of purification.

The greater part of the dioxan used was recovered in this way in a pure state.

Yield of purified fulvic acid: 140 g. from 300 flasks, *i.e.* from 5250 g. glucose.

Section D. The colourless crystalline nitrogenous compound, M.P. *132–135°, from the mycelium of* P. Brefeldianum.

On p. 1108 it is stated that the material separating from the ethereal extract obtained during the first 2 or 3 days' extraction of the mycelium was kept separate from the material from later extractions. The weight of this material (by extraction of mycelium from 300 flasks) was 41·4 g. of which some 15 g. were insoluble in aqueous $NaHCO_3$. This latter material was repeatedly crystallised from dioxan (in which it is even more sparingly soluble than fulvic acid) and was finally obtained almost colourless but not crystalline by deposition from hot ethyl acetate solution. It melted at 132–135° with slight softening from about 129° onwards, the melting point remaining unchanged after a further crystallisation from dioxan. The melt set on cooling to a turbid glass. The substance contains neither sulphur nor phosphorus. (Found (Schoeller), C, 73·35, 73·24; H, 12·42, 12·29; N, 2·1 %; Methoxyl, nil. $C_{40}H_{79}O_5N$ requires C, 73·42; H, 12·18; N, 2·1 %.) The substance is of a waxy consistency and can be obtained crystalline only from dioxan, *e.g.* from hot aqueous dioxan it separates immediately in the form of tufts of curved, microscopically small, pointed hairs; but from hot anhydrous dioxan it separates first of all in tiny oily droplets, becoming partly crystalline in 24 hours and completely crystalline after several days in the form of radiating clusters of very small, straight needles, quite similar in shape to the crystals obtained when fulvic acid separates from alcohol. It separates in tiny droplets (in appearance not unlike yeast cells) from chloroform, acetone or ethyl acetate, but these do not become crystalline even on long standing. The material separating from these solvents can however be readily collected on a Büchner funnel and in bulk is indistinguishable from the crystalline form.

The substance is moderately readily soluble in cold alcohol, but quite insoluble in boiling water. It does not give the Molisch test for carbohydrates. It gives no coloration with ferric chloride in aqueous alcoholic solution. It is quite neutral, being insoluble in both dilute acids and alkalis, even on boiling. It gives no coloration with strong sulphuric acid and appears, in fact, to be a very stable and non-reactive substance. Further work is in progress on this substance and will be reported at a later date.

Section E. Fulvic acid and its derivatives.

Properties of fulvic acid. Fulvic acid is very sparingly soluble in the cold in all organic solvents, *e.g.* at 12°, even dioxan dissolves less than 1 % of the acid, which is not sufficient for an accurate determination of molecular weight by the cryoscopic method. It may be crystallised from boiling dioxan (15 parts), acetone or ethyl acetate (100–150 parts), ethyl alcohol (250–300 parts), toluene (very sparingly soluble even at B.P.), and from water when somewhat impure, pure fulvic acid being almost insoluble in boiling water. The appearance of the crystals obtained varies with the solvent used, the rate of cooling, the amount of water present and on other factors, *e.g.* from dioxan (with or without added water), it usually crystallises in lemon-yellow rods, sometimes curved, or in small rectangular prisms, or in parallelepipeds, sometimes with bluntly pointed ends; from acetone, in massive lemon-yellow prisms; from moist ethyl acetate in relatively large golden plates; from water in pale yellow plates, characteristically twinned; from absolute alcohol in clusters of very pale yellow, slender, pointed needles; from aqueous alcohol at 0° in slender, segmented, rectangular prisms; from toluene in very small, flat crystals of indefinite shape. From acetone or ethyl acetate it sometimes happens that massive yellow prisms are found side by side with much paler slender rods. In spite of this diversity of crystalline appearance, there can be no doubt of the essential homogeneity of the product prepared in quantity as previously described, since when regenerated from the recrystallised potassium salt, the product behaves similarly when recrystallised from the above solvents. Crystals of fulvic acid tenaciously retain many solvents, and air-dried, or even vacuum-dried, specimens do not always give results in accordance with the formula $C_{14}H_{12}O_8$. Drying at 138° in a stream of dry, oxygen-free nitrogen, or in a high vacuum at 100–110° is in many cases necessary to remove the last traces of solvent. The analytical results for various specimens of fulvic acid are summarised in Table III. Fulvic acid contains no methoxyl groups and no nitrogen.

Table III.

Source and treatment	% C	% H
1. From *P. griseo-fulvum*	54·43	4·10
Crystallised from H_2O. Dried to constant weight *in vacuo* at 100°	54·41	4·02
2. From *P. flexuosum*	54·24	4·01
Crystallised from dioxan. Dried to constant weight *in vacuo* at 110°	54·09	3·98
3. From *P. Brefeldianum*		
Crystallised from toluene		
(*a*) Dried *in vacuo* at ordinary temperatures	54·44	4·06
(*b*) Dried in N_2 at 138°	54·42	4·07
Theoretical for $C_{14}H_{12}O_8$	54·53	3·93

0·1134 g. of fulvic acid from *P. flexuosum*, dissolved in 13·57 g. dioxan, gave a depression in F.P. of 0·148°; hence, molecular weight = 268 (theory for $C_{14}H_{12}O_8 = 308$). This estimation is not very satisfactory because of the slight solubility of fulvic acid in cold dioxan but full confirmation of the formula assigned, *viz.* $C_{14}H_{12}O_8$, was obtained by the preparation and analysis of the trimethyl and triethyl derivatives, and the monoethyl ester (see later).

Pure fulvic acid, after crystallisation from any solvent, always begins to darken at about 195°, becomes gradually darker as the temperature is raised, begins to soften at about 236° and melts with vigorous effervescence to a deep brown liquid at about 246°, the actual softening and decomposition points varying a little with the rate of heating. Citromycetin ($C_{14}H_{10}O_7, 2H_2O$), which shows certain resemblances to fulvic acid, melts at 285° whilst a mixture of citromycetin and fulvic acid melted at 227°. The mixed melting-points of specimens of fulvic acid produced by all three moulds were as follows: product from *P. griseo-fulvum* (crystallised from ethyl acetate) + product from *P. flexuosum* (crystallised from dioxan), softens at 238°, melts with decomp. at 244°; product from *P. griseo-fulvum* + product from *P. Brefeldianum* (crystallised from dioxan), softening 236°, decomp. 243°; products from *P. flexuosum* and *P. Brefeldianum*, softening 235°, decomp. 245°. Further confirmation of the identity of all three specimens is afforded by the mixed M.P. of their trimethyl derivatives (see p. 1113).

Fulvic acid may be sublimed in a high vacuum at a bath temperature of 170°. The yellow sublimate melts at 242–243° (decomp.) but appears to have lost a molecule of water during the sublimation. (Found on different samples (Schoeller): C, 58·01, 57·63; H, 3·70, 3·48 %. Mol. wt. (in camphor), 329. $C_{14}H_{10}O_7$ requires C, 57·92; H, 3·47 %; Mol. wt. 290).

Fulvic acid titrates as a dibasic acid but the end-point is difficult to determine accurately because of the colour of the solution. 0·1902 g. from *P. Brefeldianum* dried to constant weight in N_2 at 138° dissolved in 10 ml. dioxan + 6 ml. water required 12·39 ml. *N*/10 NaOH for neutralisation to phenolphthalein; whence equivalent = 154. ($C_{14}H_{12}O_8$ titrating as a dibasic acid requires an equivalent of 154.)

Fulvic acid is optically inactive: 0·10 g. in 15 ml. dioxan in a 1 dm. tube gave a mean reading of only +0·02°. It is soluble with difficulty in aqueous sodium acetate giving a very pale yellow solution, but is readily soluble in sodium bicarbonate (with effervescence), carbonate or hydroxide to give yellow to orange solutions. With $FeCl_3$ fulvic acid gives (*a*) a wine-red colour in absolute alcoholic solution; (*b*) a deep green in aqueous alcohol, becoming olive brown on addition of more $FeCl_3$; (*c*) a stable green colour in aqueous dioxan. Citromycetin gives a dark emerald green colour under all these conditions. Unlike citromycetin, fulvic acid does not yield a hydrobromide with HBr in glacial acetic acid, nor is a hydriodide formed when it is dissolved in hot hydriodic acid of specific gravity 1·7, since an aqueous extract of the substance crystallising out on cooling gives no precipitate with silver nitrate.

Potassium salt of fulvic acid. This salt was prepared by the method used by Hetherington and Raistrick [1931, p. 222] for the corresponding salt of citromycetin. Fulvic acid (3 g.) was dissolved in boiling ethyl alcohol (750 ml.) and to the hot filtered solution was added an excess (90 ml.) of 5 % alcoholic potassium acetate solution. The potassium salt was precipitated immediately as a yellow jelly quickly crystallising to a mass of fine needles, which after standing overnight were collected, washed with alcohol, dried and recrystallised from boiling methyl alcohol, in which it is only very sparingly soluble.

The salt as prepared above was readily but not completely soluble in water and evidently contained traces of the free acid. (Found (Schoeller) on a sample dried to constant weight in a high vacuum at 80°, loss of weight (H_2O) 9·69 %; 9·42 %. $C_{14}H_{11}O_8K, 2H_2O$ requires 9·43 % H_2O. Found on dried material, C, 48·40; H, 3·60; K, 10·21 %. $C_{14}H_{11}O_8K$ requires C, 48·52; H, 3·20; K, 11·29 %.) Hence, as with citromycetin, the salt prepared by this method is essentially a monopotassium salt.

Ethyl fulvate. Various attempts to make methyl fulvate failed. These included the following: boiling the K salt with methyl iodide in dry methyl alcohol (no action); boiling the acid with 3 % methyl alcoholic HCl (mostly unchanged); boiling with saturated methyl alcoholic HCl (no crystalline product isolated). A beautifully crystalline ethyl ester was however obtained, though in small yield, by the following method. Fulvic acid (1 g.) was boiled with saturated ethyl alcoholic HCl (150 ml.) for 2½ hours. Most of the alcohol was then distilled off, and addition of water to the residue precipitated a brown amorphous solid from which nothing crystalline could be obtained. The aqueous mother-liquor from this amorphous solid deposited a considerable amount of yellow, crystalline material overnight in the cold room. This was collected, dried and extracted with a little boiling benzene. The residue, after many crystallisations from aqueous alcohol, yielded some 30 mg. of colourless, slender, silky needles; M.P. 230–234° (decomp.), after darkening from 200° onwards, but the effervescence is not so vigorous as with the parent substance.

(Found (duplicates by independent analysts): C, 57·11, 57·31; H, 4·86, 4·98; OEt, 13·88 %. $C_{14}H_{11}O_7.OC_2H_5$ requires C, 57·14; H, 4·80; OEt, 13·41 %.) Hence the substance is monoethyl fulvate. It gives a green coloration with ferric chloride in aqueous alcoholic solution. It dissolves immediately in aqueous sodium bicarbonate to a colourless solution, and addition of HCl then precipitates the unchanged ester. Its solution in glacial acetic acid (colourless) develops a yellow colour and a greenish blue fluorescence on addition of a few drops of concentrated HCl. Concentrated HBr however produces only a yellow colour and no fluorescence. Corresponding experiments with the parent acid gave neither colour nor fluorescence.

Trimethyl derivative of fulvic acid. Fulvic acid (0·8 g.) suspended in dry ether was treated with an ethereal solution of diazomethane prepared from nitrosomethylurethane (6 ml.). Evolution of N_2 was very slow and the acid did not appear to go into solution. The reaction mixture was kept overnight and the insoluble part was then collected. It consisted of almost colourless crystals (0·6 g.), which gave no reaction with ferric chloride in alcoholic solution, and was purified by two crystallisations from benzene (200 ml.) containing a little light petroleum, and then from dioxan (2·5 ml. per 100 mg.). From either solvent it formed colourless, microscopic crystals, M.P. 192°, with effervescence and some darkening. (Found on material dried to constant weight *in vacuo* over P_2O_5 (duplicates by independent analysts): C, 58·18, 58·32; H, 5·09, 5·21; OCH_3, 26·39, 27·02 %. $C_{14}H_9O_5(OCH_3)_3$ requires C, 58·26; H, 5·18; OCH_3, 26·59 %.)

The above trimethyl derivative was prepared by the same method from specimens of fulvic acid yielded by all three moulds, and the same compound was obtained in each case as the following melting points and mixed melting points show.

A. Derivative from fulvic acid from *P. griseo-fulvum* (crystallised from aqueous dioxan): M.P. 186–188°.

B. Derivative from fulvic acid from *P. flexuosum* (crystallised from benzene-light petroleum): M.P. 188–192°.

C. Derivative from fulvic acid from *P. Brefeldianum*, described above; M.P. 192°.

Mixture of A + C: M.P. 187–190°.

Mixture of B + C: M.P. 188°.

Like fulvic acid, this derivative is sparingly soluble in all solvents. It is not sufficiently soluble in cold dioxan to allow an accurate cryoscopic determination of its molecular weight. It is slightly soluble in boiling water giving a solution with a striking blue-violet fluorescence. It gives no coloration with ferric chloride and is slowly soluble in cold dilute alkali. Addition of a few drops of concentrated HCl to the colourless solution of the trimethyl derivative in glacial acetic acid produces a pale yellow colour and an intense blue fluorescence. Concentrated HBr gives a more intense yellow colour but no fluorescence.

The same product is obtained by methylation of fulvic acid in sodium bicarbonate solution with dimethyl sulphate.

Triethyl derivative of fulvic acid. Fulvic acid (0·8 g.) was treated with ethereal diazoethane prepared from nitrosoethylurethane (6·5 ml.). The reaction was vigorous and all the material soon dissolved. After standing overnight, a colourless substance (0·5 g.) had separated. This was collected and twice crystallised from benzene (50 ml.) and light petroleum (50–100 ml.). It formed colourless aggregates of microscopic crystals; M.P. 172–174° with effervescence to a red liquid which did not reset on cooling. (Found (duplicates by independent analysts): C, 61·04, 61·28; H, 6·31, 6·36; OEt, 33·6, 35·2 %. $C_{14}H_9O_5(OC_2H_5)_3$ requires C, 61·20; H, 6·17; OC_2H_5, 34·4 %.) 0·0781 g. in 5·41 g. dioxan depressed the F.P. of the latter by 0·180°. Hence mol. wt. = 377; $C_{20}H_{24}O_8$ requires mol. wt. 392.

The properties of the triethyl derivative are very similar to those of the trimethyl derivative, save that it is more soluble in certain solvents such as benzene and dioxan. It gives no colour with $FeCl_3$. It gives a yellow colour and a brilliant blue fluorescence with concentrated HCl and glacial acetic acid.

Diacetyl derivative of fulvic acid. To a solution of fulvic acid (6 g.) in glacial acetic acid (36 ml.) were added acetic anhydride (24 ml.) and anhydrous sodium acetate (7 g.), and the mixture was boiled for 20 minutes (oil bath at 140–150°); some darkening took place. It was then cooled and poured into ice-water, but nothing separated. Acidification with dilute HCl threw down a copious, pale yellow, sandy, crystalline precipitate which was filtered off immediately (5·4 g.). The product was purified by crystallisation from alcohol, then twice from chloroform-light petroleum, then from dioxan-light petroleum and finally from aqueous dioxan. It formed very pale yellow, minute, hexagonal prisms or square tablets which did not melt at 310°, but considerable darkening and shrinking occurred above 220°. It is almost insoluble in boiling benzene and carbon tetrachloride, sparingly soluble in boiling chloroform, moderately readily soluble in boiling ethyl alcohol or cold methyl alcohol, glacial acetic acid or dioxan. Its solution in aqueous dioxan or acetic acid has a marked blue fluorescence intensified by the addition of concentrated HCl. It is immediately soluble in aqueous $NaHCO_3$ or sodium acetate to yield almost colourless solutions with a marked blue fluorescence. It gives no colour with $FeCl_3$ in aqueous alcoholic solution. (Found (Schoeller): C, 57·77, 57·85; H, 3·89, 3·79 %. Mol. wt. (cryoscopic in dioxan), 319. $C_{18}H_{14}O_9$ (*i.e.* diacetyl derivative of $C_{14}H_{10}O_7$) requires C, 57·74; H, 3·77 %. Mol. wt. 374.)

Titration: 0·2305 g. in 35 ml. methyl alcohol + 10 ml. H_2O required about 9 ml. $N/10$ NaOH for the first transient end-point to phenolphthalein, but a permanent end-point appeared to have been reached at 12·2 ml. $N/10$ NaOH. This corresponds to an equivalent of 189 (theoretical for $C_{18}H_{14}O_9$ titrating as a dibasic acid = 187). The acetyl derivative is readily hydrolysed by cold dilute NaOH as was shown in the following way: 0·1529 g. was dissolved in 20·0 ml. $N/10$ NaOH, and the solution kept at room temperature for 3½ hours; the yellow colour became much intensified. Back-titration with $N/10$ H_2SO_4 then showed that 15·5 ml. $N/10$ NaOH had been used up, corresponding with an equivalent of 99 (theoretical for $C_{18}H_{14}O_9$ titrating as a tetrabasic acid, 94). Acidification of the titrated liquid then yielded 0·10 g. of almost pure fulvic acid (theoretical, 0·12 g.).

Acetyl estimation. 0·4418 g. was boiled for 6 hours with 50 ml. N H_2SO_4. 300 ml. H_2O were then added and the liquid distilled at atmospheric pressure until only 50 ml. remained. This was repeated seven times until a constant, very small titration was obtained for volatile acid in the distillate. A net amount of volatile acid equivalent to 21·8 ml. $N/10$ NaOH was produced in all, the theoretical for $C_{18}H_{14}O_9$ yielding $2CH_3.CO_2H$ being 23·6 ml. A parallel experiment showed that fulvic acid yielded no volatile acid at all under these conditions.

It will be noticed that the empirical formula for this acetyl compound $C_{18}H_{14}O_9$ corresponds to an empirical formula for the parent substance of $C_{14}H_{10}O_7$. The empirical formula we have assigned to fulvic acid, $C_{14}H_{12}O_8$, follows from the analysis of the trimethyl and triethyl derivatives, of the monoethyl ester and of the free acid dried to constant weight at 138° in nitrogen or at 110° in a high vacuum. On the other hand, fulvic acid on sublimation in a high vacuum at 170° gives analytical results agreeing well with the empirical formula $C_{14}H_{10}O_7$. Hence we believe that fulvic acid readily loses a molecule of water under certain conditions, *e.g.* acetylation or sublimation, and we hope to find the explanation for this fact as a result of experiments at present in progress on the molecular constitution of fulvic acid.

SUMMARY.

Penicillium griseo-fulvum Dierckx, *P. flexuosum* Dale and *P. Brefeldianum* Dodge when grown on a synthetic medium containing glucose, ammonium tartrate and mineral salts all give rise to a hitherto undescribed metabolic product for which the name fulvic acid is proposed. Fulvic acid, $C_{14}H_{12}O_8$, M.P. 246° (decomp.) is a crystalline yellow pigment. The following derivatives were prepared and are described: potassium salt, monoethyl ester, trimethyl, triethyl and diacetyl derivatives.

P. griseo-fulvum and *P. flexuosum* also produce 6-methylsalicylic acid.

Two different colourless crystalline compounds containing nitrogen have been isolated—one, M.P. 165° (decomp.), from the mycelium of *P. griseo-fulvum* and the other, M.P. 132–135°, from the mycelium of *P. Brefeldianum*.

REFERENCES.

Anslow and Raistrick (1931). *Biochem. J.* **25**, 39.
van Beijma (1929). *Verhandl. Akad. Wetensch. Amsterdam, Afd. Natuurk.* [Tw. Sect.] **62**, [4], 16.
Brefeld (1874). Botanische Untersuchungen über Schimmelpilze, Heft 2. (Leipzig.)
Dodge (1933). *Mycologia*, **25**, 90.
Eigenberger (1931). *J. prakt. Chem.* **130**, 78.
Hetherington and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 209.
Lockwood, Ward, May, Herrick and O'Neill (1934). *Z. Bakt.* **90**, 411.
Oxford (1934). *Biochem. J.* **28**, 1325.
—— and Raistrick (1933). *Biochem. J.* **27**, 1176.
—— —— (1934). *Biochem. J.* **28**, 1321.
Raistrick and Simonart (1933). *Biochem. J.* **27**, 628.
Shear (1934). *Mycologia*, **26**, 104.
Thom (1930). The Penicillia. (Baillière, Tindall and Cox, London.)

CXL. STUDIES ON THE ANAEROBIC DECOMPOSITION OF PLANT MATERIALS.

III. COMPARISON OF THE COURSE OF DECOMPOSITION OF RICE STRAW UNDER ANAEROBIC, AEROBIC AND PARTIALLY AEROBIC CONDITIONS.

BY CADAMBI NARASIMHA ACHARYA.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden, Herts.

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THE experiments reported in this paper form a continuation of those already described [Acharya, 1935, 1, 2] and comprise a comparative study of the rate and course of decomposition of rice straw under anaerobic, aerobic and partially aerobic (*e.g.* water-logged) conditions.

The procedure and analytical details adopted were the same as those previously reported except for the modifications noted below. Ammonium carbonate equivalent to 1 % N on the straw, 0·5 g. K_2HPO_4, 0·05 g. $MgSO_4$, $7H_2O$ and 10 ml. of an extract from a rice soil were added to 20 g. portions of chaffed rice straw. The aerobic decomposition was carried out in open dishes, keeping the mass at a straw : water ratio of about 1 : 4 by additions of water from time to time. Water-logged conditions were obtained by having about an inch of water above the straw which was kept in beakers. Every few days the mass was stirred and fresh water added to replace evaporation losses. Decomposition under anaerobic conditions was carried out in stoppered bottles as already described [1935, 1, 2]. It was observed that abundant fungal growth accompanied decomposition under aerobic conditions and that even under water-logged conditions a thin fungal layer formed on the surface of the water when the mass was not stirred.

In addition to the above comparisons the effect of a regulated admission of air was tested by incubating samples in stoppered bottles and bubbling air through them at weekly intervals, after the accumulated gases had been tapped off for analysis. In one set of bottles a current of air was drawn for 5 minutes through the bottles just over the surface of the liquid; this is termed "mild aeration." In another set, air was bubbled through the liquid for 5 minutes so as to replace the dissolved gases by air and this is termed "strong aeration."

The results obtained are given in Table I. They show that the rate of decomposition is highest under aerobic conditions and lowest under anaerobic conditions, the partially aerobic (*e.g.* water-logged) systems occupying an intermediate position. At the end of 6 months the losses in ash-free insoluble dry matter are 65·0, 47·5 and 55·5 % for aerobic, anaerobic and water-logged fermentations respectively. The relative ease with which the cellulose and hemicellulose fractions of straw are attacked varies, however, in the different cases. Under anaerobic conditions, the hemicelluloses are the first to be attacked; at the end of one month 26·8 % of the amount originally present has been decomposed as against 9·8 % of the cellulose. At the end of the same period under aerobic conditions the loss of hemicelluloses is 62·4 % and of cellulose 56·2 %; the water-logged straw loses 38·0 % of hemicelluloses and 32·0 % of cellulose. The water-logged system thus resembles the aerobic system in the relative proportions of cellulose to hemicellulose destroyed, though the actual losses are much lower in the former case. The greater loss of cellulose in the early stages in these two systems, as compared with the anaerobic system, is probably due to the activity of cellulose-decomposing fungi.

The greatest loss of lignin occurs in the water-logged straw, whether determined on the material without any treatment or after a preliminary hydrolysis with 5 % H_2SO_4 [Norman and Jenkins, 1933, p. 826]; the losses are 39·6 % and 33·1 % of the initial value by these two methods. The corresponding losses under anaerobic conditions are 28·5 % and 28·5 %, and under aerobic conditions 33·5 % and 29·8 % respectively.

A striking difference between the three methods of fermentation lies in the protein content of the insoluble residue. In the aerobic system this rises from 2·88 g. per 100 g. straw at the start to 5·45 g. at the end of 6 months, whereas under anaerobic and partially aerobic conditions losses of this constituent are found. Of these two latter residues, the anaerobic contains less protein (2·01 g.) than the water-logged (2·69 g.); this difference is probably associated with the more intense reducing conditions present in the anaerobic system which help to bring more of the protein into solution.

The loss of added ammonia is most rapid under aerobic conditions, about 46 % of it being lost by volatilisation in a fortnight. The loss is less rapid from the water-logged straw, about 18 % being lost during the same period. The added ammonia is much better conserved under anaerobic conditions, the loss over a period of 6 months amounting to only 15 %; this no doubt is due to the closed nature of the system and the limited number of occasions on which the accumulated gases are let out for analysis.

The differences in the nature of the products obtained under aerobic and anaerobic conditions have been dealt with in a previous communication [1935, 1]. The formation of organic acids and the evolution of combustible gases characteristic of anaerobic decomposition are absent in the former case, where intermediate products if any are rapidly oxidised to CO_2. Water-logged conditions resemble the anaerobic system in the rapid accumulation of organic acids in the early stages, but these are decomposed much more rapidly than in the latter case.

The influence of partial aeration on anaerobic decomposition is shown in Tables I and II under the heads "mild aeration" and "strong aeration." The significance of these terms has been explained already.

With "mild aeration" the anaerobic conditions in the liquid portion are not appreciably disturbed as shown by the accumulation of organic acids and the evolution of methane and hydrogen. There is a marked increase in the amount of CO_2 evolved, presumably due to the admission of air, and a decrease in the amount of CH_4 formed. The amount of H_2 evolved shows a great increase as compared with anaerobic conditions. This difference, it is suggested, is associated with the oxidation-reduction potential of the system under these conditions.

The decomposition with "strong aeration" resembles that under aerobic conditions as shown by the small amount of organic acids, CH_4 and H_2 formed and the large amounts of CO_2 evolved (Table II). The high proportion of free nitrogen contained in the gases evolved in both aeration treatments is derived from the air passed through the bottles.

The nitrogen relationships under the different treatments are of special interest. It has already been noted that the amount of protein in the insoluble residue is lowest in the anaerobic system and highest in the aerobic system.

Table I. *Decomposition of rice straw at 30° under aerobic, anaerobic and partially aerobic conditions.*

	100 g. original straw g.	Aerobic				Anaerobic				Partially aerobic					
										Water-logged				Mild aeration	Strong aeration
		2 weeks g.	1 month g.	3 months g.	6 months g.	2 weeks g.	1 month g.	3 months g.	6 months g.	2 weeks g.	1 month g.	3 months g.	6 months g.	6 months g.	6 months g.
						Losses of constituents.									
Dry residue less ash	74·1	24·1	35·7	45·4	48·2	10·2	15·2	22·9	35·2	18·2	24·5	32·2	41·1	36·8	34·4
Cellulose (including xylan)	44·55	17·25	25·05	33·01	35·25	2·12	4·38	10·47	21·26	10·20	14·25	19·20	25·95	24·60	23·60
Xylan in cellulose	11·73	4·28	6·38	8·88	9·93	0·91	1·54	3·25	6·36	2·08	3·18	4·98	7·23	6·03	5·93
Total xylans	21·04	9·59	13·14	17·44	18·24	4·12	5·64	8·78	12·79	5·09	7·99	11·59	15·44	13·04	11·99
Lignin (direct)	10·67	1·52	2·77	3·37	3·57	0·72	0·87	1·47	3·04	1·75	2·27	3·47	4·22	2·63	2·93
Lignin (after hydrol.)	9·12	1·37	1·97	2·37	2·72	0·61	1·20	1·99	2·60	1·58	2·02	2·77	3·02	2·22	2·67
Protein	2·88	+1·35	+1·83	+2·25	+2·57	0·82	0·87	0·87	0·87	0·15	0·16	0·18	0·19	0·28	0·19
						Present in aqueous extract.									
Organic acids	—	Nil	Nil	Nil	Nil	6·42	7·50	7·26	1·30	10·82	3·58	1·50	0·12	7·38	0·73
Protein-N	—	0·050	0·075	0·100	0·125	0·135	0·153	0·138	0·207	0·315	0·350	0·375	0·425	0·310	0·360
Nitrate-N	—	0·025	0·050	0·050	0·050	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
NH_4-N	—	0·275	0·125	0·050	Nil	0·824	0·812	0·781	0·704	0·525	0·210	0·095	0·040	0·651	0·515
p_H of medium	—	8·8	9·0	9·2	9·2	7·0	7·2	7·2	7·8	7·0	7·4	8·2	8·8	7·2	8·8
						Nitrogen relationships.									
Protein-N in residue	0·461	0·677	0·754	0·821	0·872	0·330	0·320	0·324	0·323	0·438	0·435	0·432	0·431	0·416	0·431
Protein-N in aq. extract	—	0·050	0·075	0·100	0·125	0·135	0·153	0·168	0·207	0·315	0·350	0·375	0·425	0·310	0·360
Total protein-N present	—	0·727	0·829	0·921	0·997	0·465	0·473	0·492	0·530	0·753	0·785	0·807	0·856	0·726	0·791
N immobilised or nitrogen factor	—	0·266	0·368	0·460	0·536	0·004	0·012	0·031	0·069	0·292	0·324	0·346	0·395	0·265	0·330
Nitrogen equivalent	—	1·10	1·03	1·02	1·11	0·039	0·079	0·135	0·196	1·604	1·322	1·074	0·961	0·701	0·932

Table II.

Per 100 g. straw	Anaerobic ml.	Mild aeration ml.	Strong aeration ml.
CO_2 evolved	10,850	17,405	32,500
CH_4 evolved	10,570	6,880	2,130
H_2 evolved	105	5,625	925
N_2 evolved	85	12,155	14,205

Whereas under anaerobic and water-logged conditions increasing amounts of protein dissolve with increasing periods of fermentation, in the aerobic system there is a continued accumulation of protein in the residue, due to the synthesis of insoluble microbial tissue.

The water-soluble protein-nitrogen is at a minimum under aerobic conditions (0·125 g. per 100 g. straw), as compared with anaerobic (0·207 g.) and water-logged systems (0·425 g.). The high value for soluble protein-nitrogen under water-logged conditions is noteworthy since most of it is synthesised from the ammonia originally added. It is an index of the great microbial activity under these conditions and is in marked contrast to the poor nitrogen immobilisation under anaerobic conditions.

The figures for nitrogen factor [Rege, 1927] and nitrogen equivalent [Richards and Norman, 1931] also show striking differences between the three sets of conditions. They are highest under aerobic conditions being 0·536 and 1·11 respectively, followed by water-logged conditions (0·395 and 0·961 respectively), while the anaerobic system shows the minimum values (0·069 and 0·169 respectively). Even "mild aeration" favours protein synthesis as shown by the jump of the nitrogen factor from 0·069 to 0·265, while "strong aeration" increases it to 0·330.

DISCUSSION.

The results indicate that partially aerated systems show the characteristics of both the aerobic and anaerobic types of decomposition of straw and that the degree of aeration decides which type predominates. Thus "mild aeration" resembles the anaerobic system because of the organic acids and methane formed, with the important difference that under suitable conditions a large amount of hydrogen is also liberated. More CO_2 is evolved than under anaerobic conditions. With "stronger aeration," conditions approach the aerobic system as shown by the small amount of organic acids, CH_4 and H_2 produced and the large amount of CO_2 evolved. Water-logged conditions occupy an intermediate position between the above two systems.

The water-logged system resembles the anaerobic system in some respects, *viz.* in the formation of organic acids, liberation of methane and formation of soluble protein, but in others it shows differences, *e.g.* in the relative losses of cellulose, hemicelluloses and lignin and in the synthesis of protein from ammonia. These differences could be explained by the presence of a wider variety of micro-organisms in the water-logged system able to attack cellulose and lignin. Moreover, the lower reducing power under water-logged conditions, as compared with the anaerobic system, permits an accumulation of proteins synthesised as a result of microbial activity. In these respects the water-logged system more closely resembles the aerobic system.

The admission of even limited amounts of air at weekly intervals alters the nitrogen relationships of the anaerobic system, as shown by the synthesis of protein from ammonia and by the rapid increase in the values for nitrogen factor and nitrogen equivalent.

SUMMARY.

1. The relative rates of decomposition of rice straw in decreasing order are: aerobic, water-logged and anaerobic fermentations. Cellulose and lignin are more easily attacked under water-logged conditions than under anaerobic conditions.

2. Partially aerobic conditions resemble anaerobic conditions in regard to the nature of the products obtained. Smaller amounts of organic acids and methane are, however, formed and there is an increase in the amount of CO_2 evolved. Under conditions of mild aeration much larger quantities of hydrogen are obtained than under anaerobic conditions.

3. The nitrogen factor and nitrogen equivalent decrease in the order: aerobic (0·536; 1·11), water-logged (0·395; 0·961) and anaerobic (0·069; 0·169), showing the degree of protein accumulation in the three cases. The protein formed under aerobic conditions is mostly insoluble in water, while under water-logged conditions it remains in solution.

4. In most respects the decomposition of rice straw under water-logged conditions gives results intermediate between aerobic and anaerobic decompositions.

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REFERENCES.

Acharya (1935, 1). *Biochem. J.* **29**, 528.
—— (1935, 2). *Biochem. J.* **29**, 953.
Norman and Jenkins (1933). *Biochem. J.* **27**, 818.
Rege (1927). *Ann. Appl. Biol.* **14**, 1.
Richards and Norman (1931). *Biochem. J.* **25**, 1769.

CXLI. STUDIES ON THE VITREOUS BODY. II.

BY WILLIAM STEWART DUKE-ELDER AND HUGH DAVSON.

*From the Department of Physiology and Biochemistry,
University College, London.*

(Received March 22nd, 1935.)

IN an earlier paper [Duke-Elder *et al.*, 1935] direct experiments were described showing that the vitreous body (henceforth called simply the vitreous) does not take up water *in vitro* under the influence of soaps and changed p_H. As the clinical picture of primary chronic glaucoma strongly suggests that a swelling of the vitreous is closely related to the raised intra-ocular pressure the work was continued with a view to showing whether such a change in volume of so dilute a gel can reasonably be expected on physico-chemical grounds.

So far two possible theories of the mechanism of volume increase have occurred to us depending on different conceptions of the mode of origin of the vitreous. First, if it is assumed that the vitreous is formed by a continuous process of swelling from an initial xerogel similar to the process of swelling of dry gelatin in water, then, considering it as at its swelling maximum at normal p_H, it seems possible that it could swell further if the p_H were altered in a direction favourable to increased hydration.

All previous experiments designed to show whether the vitreous swells have tacitly assumed the truth of the latter proposition. However, although the colloid chemical literature contains many studies on the swelling of gels in the presence of foreign substances, no one, it seems, has studied the influence of these substances on a gel already at its swelling maximum. The procedure in all cases has been to swell a quantity of the xerogel at one p_H, say, and then to take another lot of the xerogel and swell it at a new p_H and so on. On consideration it is by no means evident that a swollen gel will respond to a change in its environment to the same extent or in the same way as the dry xerogel, owing to the intervention of peptisation which is particularly to be expected when the micelles are already strongly hydrated.

If, on the other hand, the vitreous is formed by a sol to gel change another theory must be presented to show how it can swell[1]. In this case it is suggested that a minute fraction of the vitreous is peptised owing to the presence of some lyophilic substance; if, then, the procedure is reversed whereby the comparatively protein-rich fluid is converted into its gel form, the presence of the adsorbed hydrotropic substance enables the protein to orient more water than previously. The net result is an increase in volume. If this were to proceed over a number of years the effect would be to produce the pressure symptoms characteristic of chronic glaucoma. The process may be illustrated in a crude way by placing a lump of 10 % gelatin gel in a small volume of water. Addition of acid produces eventual peptisation; neutralisation of the acid converts the sol back into a gel of larger volume than before. (The objection that the increased pressure will expel fluid after the peptisation of a portion of the vitreous is overcome by postulating a unidirectional permeability of the surrounding membranes. This is not a further *ad hoc* assumption, as some such state must be the case or the vitreous would break down under the unbalanced stress.)

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[1] Unpublished experiments of our own on gelatin clots together with theoretical considerations lead to the conclusion that a dilute clot, as opposed to a swollen gel, will not increase in volume directly when its environment is changed.

This paper is divided into three parts according as the experiments described throw light on the possibility of a gel at its swelling maximum swelling further on changing the p_H suitably (Part I), on the structure of the vitreous and hence its mode of origin (Part II), and on the possibility of peptising the residual protein (Part III).

Experimental.

Part I. Swelling of fibrin.

Pure fibrin, prepared by the combined methods of Fischer [1908] and Schuldenzucker [1928], was swollen at different p_H values in the acid range in a Schmidt apparatus [1931] which permits measurements of water uptake to within 0·002 ml. In Fig. 1 the water uptake per g. of dry fibrin is plotted against p_H. It is evident that by allowing fibrin to swell to its maximum at p_H 6 and then changing the medium to one of p_H 2 the conditions necessary to show whether a gel at its swelling maximum can take up further water by changing the p_H favourably will be achieved.

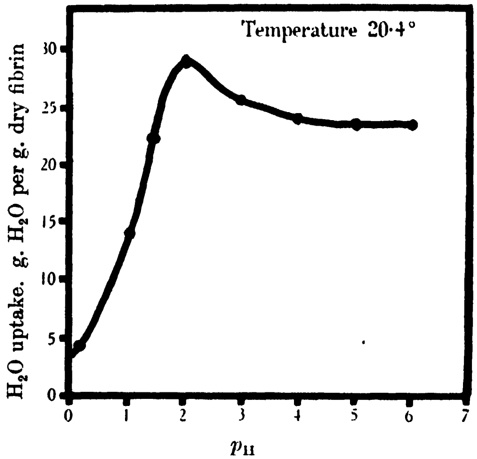

Fig. 1.

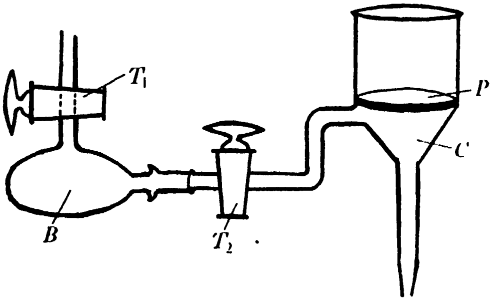

Fig. 2.

To this end the Schmidt apparatus was modified as in Fig. 2. A bulb *B* with a tap T_1 fits on the side-tube by a ground joint. With T_2 closed the bulb is filled with concentrated HCl and T_1 is closed. Fibrin is placed on the porous plate, *P*, and when the water uptake, as measured by the movement of a meniscus in the capillary in communication with the chamber, *C*, has reached its maximum the tap T_1 is opened allowing acid to diffuse in, thus lowering the p_H of the fluid in contact with the swollen fibrin.

In Fig. 3 the water uptake is plotted against time. For convenience the initial part of the curve is not shown and the scale of ordinates above 2·8 ml. is half of that below this value. Obviously the swollen fibrin adjusted itself to the changed p_H. If the acid had not diffused in, the water uptake would have reached a final value of 2·80 ml., whereas, owing to the changed acidity, it rose to a maximum of 3·18 ml.

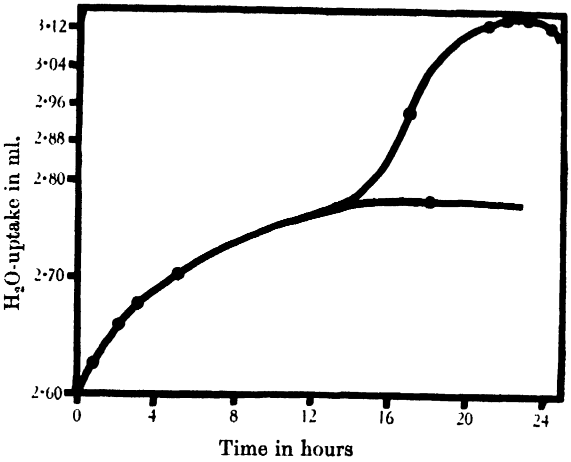

Fig. 3.

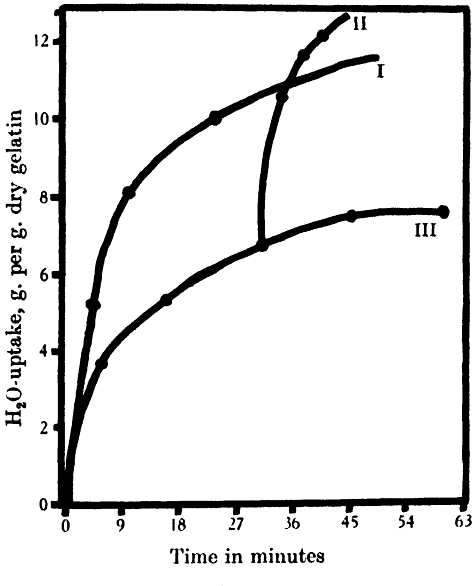

Fig. 4.

Swelling of gelatin. Gelatin was allowed to swell at p_H 4·8, the isoelectric point and hence the p_H at which swelling is minimum, and at p_H 1. Pieces of gelatin which had already swollen at p_H 4·8 were transferred to the solution of p_H 1 and their subsequent swelling followed.

Coignet's leaf gelatin was cut into approximately equal squares and after drying over conc. H_2SO_4 placed in a dish containing 2 l. of the solution in a room maintained at constant temperature (20·4°). At intervals pieces were removed, blotted with filter paper, and placed on weighed pieces of filter-paper which had been previously dried over H_2SO_4. The filter-paper and swollen gelatin were weighed and placed in a vacuum desiccator. After 24 hours drying was completed over H_2SO_4 and they were weighed again. The weight of H_2O taken up per g. of dry gelatin was plotted against time, Fig. 4.

Although a cruder technique than that of Jordan-Lloyd [1927] this has the advantage that the H_2O content of the swollen gel as it is in its final state is determined, whereas Jordan-Lloyd, by weighing the initial quantity of dry gelatin and then that of the wet gelatin remaining, is able to tell us nothing more than the change in weight of a piece of gelatin after placing it in a given medium. A medium very favourable to swelling dissolves a considerable portion of the gelatin so that by her technique the swelling of gelatin would appear to fall off with increasing acidity. This is definitely not the case.

In Fig. 4 I and II are the swelling curves of gelatin in solutions at p_H 1·1 and 4·8 respectively. The upper branch of curve II represents the change in the rate of swelling on transferring the gelatin from a medium of p_H 4·8 to one of p_H 1·1. It is at once evident that there is an immediate adjustment to the changed p_H.

Part II.

Goedbloed [1934, 1] described ultramicroscopic changes in the vitreous on treatment with KCNS solutions, which he interpreted as being due to an increased hydration of the micelles. If such were the case a change in the elastic properties of the vitreous on treatment with this substance would be expected. To test this two techniques were used, first the resistance of the treated vitreous to the emulsification of Hg by ultrasonic waves was compared with that of a control, and secondly the technique of Freundlich and Seifriz [1923] and Seifriz [1924] for the measurement of the elastic properties of gels, as modified by Robertson and Duke-Elder [1933, 1] to suit the peculiarities of the vitreous, was used.

Ultrasonic waves. When H_2O and Hg are submitted to ultrasonic waves, an emulsion of Hg in H_2O is immediately formed, the whole mixture taking on a dark grey colour [Wood and Loomis, 1927; Bull and Söllner, 1932]; if the H_2O is replaced by a jelly the elastic properties of the latter hinder the emulsification of the Hg so that after a given time there is a fairly well-defined boundary between the clear jelly and that into which Hg particles have penetrated. The height of this boundary gives a rough idea of the elasticity of the jelly.

Ox vitreous was placed in 1·0 *M*, 0·5 *M* and 0·14 *M* solutions of KCNS and in isotonic saline for 24 hours at 20°. The samples were transferred to test-tubes and 1 ml. of Hg added, and the tubes were held in succession over the vibrating column of oil above the quartz plate of the ultrasonic apparatus. The heights of the emulsified Hg columns were compared.

There was no detectable difference in the heights of the columns of the vitreous treated with KCNS and of those treated with normal saline, so that it is concluded no very gross change in the elastic properties of the vitreous are caused by KCNS.

Nickel particle technique. Vitreous is placed in a rectangular glass cell, 1 cm. edge, and a nickel particle about 50μ diameter is picked off a slide under the microscope and stabbed into the vitreous. The particle is focused and its extension due to an applied magnetic field measured by a micrometer eye-piece. Portions of vitreous treated with KCNS, K_2SO_4 and dilute HCl at p_H 3 were studied together with normal untreated vitreous. The results are shown in Figs. 5 and 6.

Curve 1, Fig. 5, is the typical extension-time curve for normal untreated vitreous, characterised by a rapid initial elastic displacement followed by a viscous creep approaching a fairly constant velocity.

Before describing the remaining curves we must say a few words in regard to the limitations of the technique and the results expected should hydration or dehydration of the colloid particles be achieved by the solutions mentioned. Firstly, a direct comparison of the absolute displacements of one given particle in different lots of vitreous in a given time and with a fixed field strength is difficult, as a particle of the size necessary for its recovery from the first vitreous is very irregular and its extension will depend on the direction of motion. Furthermore, as the vitreous is not stuck firmly to the cell walls there will be a certain amount of "give" which will vary with the quantity of vitreous in the cell and the depth of the particle in it. On the other hand the curve I, Fig. 5, is very typical and can be reproduced from vitreous to vitreous without difficulty. As far as the changes expected on treatment of the vitreous with the reagents mentioned are concerned, from the work of Leick [1904] and Kramer [1926] a decrease in elasticity is to be expected with K_2SO_4 and an increase with KCNS. This should be reflected in a diminution in the viscous creep, characterised by the upper part of the curve, in relation to the initial elastic displacement, in the case of K_2SO_4, and the reverse should hold for KCNS. (Once again no work has been done on the effect of such solutions on gels already formed; workers in this field have added the substance to be examined to the sol which was then allowed to set, and the elastic modulus measured.)

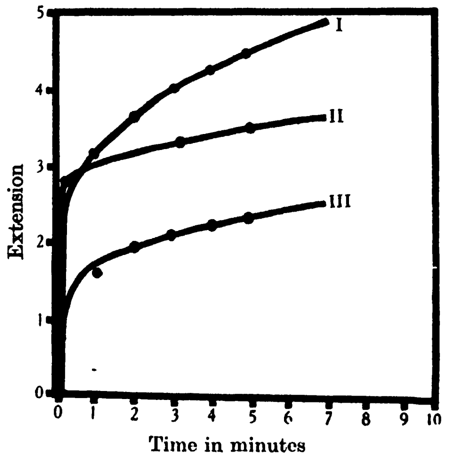

Fig. 5.

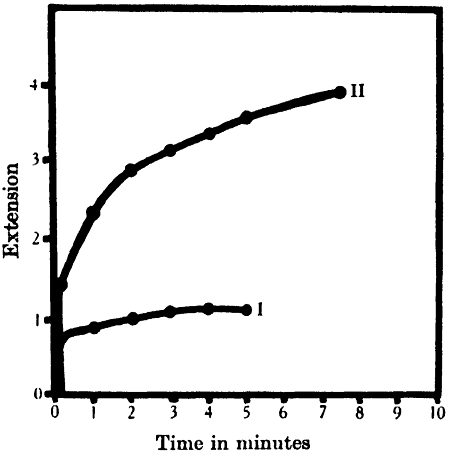

Fig. 6.

Curves II and III of Fig. 5 are those for vitreous treated with 0·5 *M* KCNS and K_2SO_4 respectively. They are representative of a number of curves determined similarly, but it should be mentioned that a few curves were obtained not conforming to these types; the reason for these irregularities, which were not observed in normal vitreous, will be brought out later. The displacements in the different curves are in no way comparable with one another as different particles were used. It is evident that 0·5 *M* K_2SO_4 has no appreciable effect on the extension-time curve of ox vitreous, whilst 0·5 *M* KCNS has the reverse effect to that expected on the supposition that it increases hydration, the extension subsequent to the first 10 secs. being very small compared with that during this time. The absence of effect by 0·5 *M* K_2SO_4 is very surprising as a considerable contraction in volume was observed. The curves for isotonic solutions of these salts (not shown) were in general similar to those for normal vitreous.

The curves obtained with vitreous treated with dilute HCl at p_H 3 (Fig. 6) throw a little light on the problem. Curve I was obtained by applying a small magnetic force; increasing the field with conditions otherwise the same gave curve II which is quite normal. It is evident that the viscosity of the medium has increased under the influence of the acidity, so that a limiting force has to be exerted to allow the creep to proceed. A similar phenomenon was observed with K_2SO_4 but not so regularly.

Baurmann [1924] showed that between p_H 3 and p_H 2 a precipitate fall in the volume of the vitreous occurs; this change is accompanied by the precipitation of the mucoprotein in the gel. If we assume that the volume change is due to the collapse of the vitreous structure, as opposed to a semi-reversible dehydration, then at p_H 3 we should expect the undermining of the vitreous structure to have already begun, and hence the changes in the elasticity could be put down to this cause. The decreased stability of the vitreous, which would tend to lower the elasticity, would at the same time increase the viscosity of the intermicellar fluid and the two effects would compensate each other to varying extents. If this is true, the irregularities in the shapes of the curves obtained by treating the vitreous with KCNS, K_2SO_4 and dilute HCl mentioned earlier are explained, as it is only to be expected that a body in a state of very unstable equilibrium will respond extravagantly to minute changes in its environment, thus prejudicing the reproducibility of results obtained on it.

In view of the results obtained on the extension-time curves, it was important to see whether the absolute values for the extension of a given particle varied greatly according as the vitreous was treated with K_2SO_4, KCNS or dilute HCl. The only way of achieving this end was to treat a large number of pieces of vitreous successively and analyse the results statistically.

Two cells were filled at the same time to the same height with normal and treated vitreous. A particle was placed in one, and its extension in a fixed magnetic field lasting for 5 secs. was measured. Immediately the particle was recovered and placed in the other cell, and its extension under the same conditions measured. This was repeated for 12 to 13 pairs and the results are tabulated in Table I.

Table I.

Solution	*d*	*n*	*s*	*t*	*P*
0·5 *M* KCNS	+4·34	13	2·56	3·3	<0·01
0·5 *M* K_2SO_4	−1·10	12	4·21	0·9	0·3–0·4
$1 \cdot 10^{-3}$ *M* HCl	−2·30	13	2·53	3·3	<0·01

In Table I *d* is the mean difference in extension for normal and treated vitreous; a positive value means that the treated vitreous gave a larger extension than its control. *n* is the number of pairs of determinations, *t* is the ratio of the mean difference to its standard error [Fisher, 1928], and *P* is the probability that such a value of *t* will occur by chance. It appears that in 0·5 *M* KCNS the extension is greater and in dilute HCl at p_H 3 less than the control whilst the difference with K_2SO_4 is insignificant. Here again the results are more in conformity with the supposition that the vitreous stability has been upset than that a reversible syneresis has occurred, or in the case of KCNS a reversible hydration. The relation of these results on the elasticity of the vitreous to its mode of origin will be dealt with in the discussion later.

As the volume decrease of the vitreous on treatment with dilute HCl appeared to be correlated with the precipitation of its mucoprotein [Goedbloed, 1934, 2] we decided to determine in what way the presence of a second protein influenced the stability of a protein clot.

Pure de-ashed gelatin was dissolved in phosphate buffer at p_H 7 to 10 % and 5 ml. lots were transferred to test-tubes. 2 ml. of a protein solution in phosphate buffer at the same p_H were added to one group and 2 ml. of the phosphate solution to another which served as controls. After equilibrating in a bath at 38° pairs were transferred to another at 20° and the course of setting followed by dropping in lead shot at intervals. When a shot failed to penetrate the surface the solution was considered to have set. As Fraas [1894] has shown that at room temperature complete equilibrium is only attained after 24 hours, the test-tubes were transferred after setting to the ice-chest overnight and the melting times of the clots determined by placing them in the bath at 38°. The results are shown in Table II; positive signs indicate degrees of increased stability of the clots as evidenced by a more rapid setting or a more protracted melting.

Table II.

Protein solution added	Change in stability	
	Setting time	Melting time
1 % ovalbumin	———	+
0·5 % agar-agar	———	+
2·0 % agar-agar	+ + +	+ + +
1·0 % serum globulin	+	−
0·75 % isinglass	———	+ +
1·5 % isinglass	———	+ +
2·0 % caseinogen	− −	− −

It is evident from Table II that a second protein, or in the case of agar-agar a carbohydrate, may enter into the intimate structure of a gelatin gel. Hitherto, in the vitreous, the residual protein has been considered as the determining factor in the degree of hydration of the vitreous. The results given in this paper lead to the view that the vitreous stability and hydration are bound up with the physical states not only of the residual protein but also of the mucoprotein and perhaps of the albumin and globulin.

Part III. Attempts at peptisation of the residual protein.

The vitreous was submitted to ultrasonic vibrations[1], as a class of gels, the thixotropic gels, are converted into their sol form by this treatment. No liquefaction of the vitreous was obtained in this manner. Solutions of KCNS of strengths varying from 0·5 *M* to a saturated solution, a variety of soaps, and thiourea, in the heat and the cold, were tried on the separated residual protein without any evidence of peptisation. Mörner's treatment [1894] by which the protein was heated with H_2O at 105° under pressure gave a clear solution, which, however, failed to set on cooling as described by him. Repeated attempts using this treatment failed to give a jelly, and it must be concluded that this vigorous treatment gave breakdown products.

DISCUSSION.

In accordance with the results of Part I, it is probable that if the vitreous were formed by a continuous process of swelling without the intervention of a sol stage, then the presence of soaps in the surrounding tissues would favour an increased volume of the vitreous (as suggested in the first paper of this series).

The results of Part II, however, in so far as they show that the effects of acid, KCNS and K_2SO_4 tend to lower the stability of the gel, point to the vitreous having been formed as a clot from an aqueous solution of the proteins, since gels formed by a swelling process alone exhibit a certain degree of reversibility in their hydration and dehydration phenomena. When, furthermore, the similarity in the elastic and ultramicroscopic properties of the vitreous and dilute plasma clots is considered [Robertson and Duke-Elder, 1933, 2] the balance of evidence points to the vitreous having been formed as a clot. (The histological study of embryonic vitreous development tells us very little about its physical mode of origin. In a preparation using Zenker's solution as a fixative streaks are seen emerging from the epithelium of the optic cup and later from the glial elements of the retina.) The experiments with gelatin clots show that it is likely that the mucoprotein and albumin and globulin of the vitreous enter intimately into the structure of this body.

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[1] We are indebted to Prof. Freundlich and Dr Söllner for performing the experiments involving the use of ultrasonic waves described in this paper.

The unsuccessful attempts at peptising the residual protein described in Part III lend a certain support to the idea of a swelling origin of the vitreous, and militate against the theory that the raised intra-ocular pressure in glaucoma is brought about by a cyclical process of peptisation and clotting. Attention should be drawn here to the statement by Goedblocd [1934, 1] that KCNS in concentrations of 2·5 M and greater causes a complete solution of the vitreous. Such is not the case; we have observed repeatedly that, whilst at 2 M concentration and greater there is a decrease in the volume of the vitreous culminating at a concentration of 2·5 M in its shrinkage to a minute fraction of its original volume, the effect is not one of solution but one of dehydration as the residual protein is to be seen invariably floating on the top of the solution. We may add that the context of Goedbloed's statement leaves no doubt that it was his intention to convey the idea that the residual protein completely dissolved. The same author [1934, 1, 2] denies that the vitreous is a gel on the grounds that the phenomena associated with it are not reversible. There is no point in discussing the matter at length: we can only say here that his exclusion of the vitreous from the category of gels on this count simultaneously rules out practically all substances hitherto considered as belonging to this class.

SUMMARY.

The physico-chemical mode of origin of the vitreous body, the possibility of its swelling *in vivo*, and factors in its stability and structure are investigated.

The subsequent behaviour of swollen gels on altering the p_H is described.

The changes in the elastic properties of the vitreous after treatment with solutions of K_2SO_4, KCNS and dilute HCl are examined, leading to the conclusion that a decrease in stability, as opposed to reversible changes in hydration, is the consequence of these treatments.

The influence of proteins and agar-agar on the setting and stability of gelatin gels is studied.

It is concluded that the mucoprotein enters intimately into the vitreous structure and a possible mechanism of an increase in the volume of the vitreous based on a cyclical process of peptisation and clotting is suggested.

All attempts at peptising the vitreous or its isolated residual protein were without success.

We wish to thank Profs. Evans and Drummond for their valuable interest in this work, and the Medical Research Council for defraying the whole expenses of this research and for a personal grant to one of us (H. D.).

REFERENCES.

Baurmann (1924). *Arch. Ophthal.* **114**, 280.
Bull and Söllner (1932). *Kolloid Z.* **60**, 263.
Duke-Elder, Robertson and Davson (1935). *Biochem. J.* **29**, 72.
Fischer (1908). *Pflüger's Arch.* **124**, 69.
Fisher (1928). Statistical methods for research workers.
Fraas (1894). *Wied. Ann.* **53**, 1082.
Freundlich and Seifriz (1923). *Z. physikal. Chem.* **104**, 233.
Goedbloed (1934, 1). *Arch. Ophthal.* **132**, 323.
—— (1934, 2). *Arch. Ophthal.* **133**, 1.
Jordan-Lloyd (1927). *Biochem. J.* **21**, 1352.
Kramer (1926). *Coll. Symp. Mon.* **4**, 102.
Leick (1904). *Ann. Phys.* (4) **14**, 139.
Mörner (1894). *Z. physiol. Chem.* **18**, 253.
Robertson and Duke Elder (1933, 1). *Proc. Roy. Soc. Lond.* B **112**, 215.
—— —— (1933, 2). *Proc. Roy. Soc. Lond.* B **112**, 224.
Schmidt (1931). *Z. physikal. Chem. Bodenstein Festband*, 333.
Schuldenzucker (1928). *Z. Biol.* **87**, 269.
Seifriz (1924). *Brit. J. Exp. Biol.* **2**, 1.
Wood and Loomis (1927). *Phil. Mag.* (7), **4**, 417.

CXLII. THE REDUCTION POTENTIALS OF BACTERIAL SUSPENSIONS.

BY JOHN YUDKIN[1].

From the Colloid Science and the Biochemical Laboratories, Cambridge.

(*Received March 23rd, 1935.*)

A NUMBER of observations have been recorded on the reduction potentials of growing bacteria [Gillespie, 1920; Cannon *et al.*, 1926; Aubel and Aubertin, 1927; Aubel and Genevois, 1927; Hewitt, 1930, 1, 2, 3]. It was considered that, in this earlier work, the continuous changes occurring in the medium during the growth of the organisms complicated the problem to an extent which made it impossible to obtain a clear idea of the cause of the production of the potentials recorded. In order to simplify the system, the following work was carried out with washed suspensions instead of with growing organisms.

In a few cases measurements on washed cells have already been carried out. Thus, Aubel *et al.* [1928] have recorded the potentials of kidney, liver and muscle tissue, and Cannon *et al.* [1926] measured the potentials of washed yeast cells. These latter authors find a slow fall of potential, which becomes much more rapid after the addition of succinate. If methylene blue is added after these low values have been reached, the potential is brought up to the range characteristic of the dye until the dye is reduced. This reduction is very slow, and the potential correspondingly does not soon fall, in the absence of succinate, whereas in its presence this "poising" action of the dye is soon overcome and the potential rapidly resumes its negative values.

In none of this work has the actual cause of the potentials been made clear. It is conceivable that they are due to the cells themselves influencing the electrode. In this case, the increased negativity observed on the addition of metabolites might be due to some combination between the enzymes of the cell and the metabolites which is capable of affecting the electrode to a greater degree than the cells alone. On the other hand, the electrode potential may be due to substances formed by the cell enzymes, which substances are themselves capable of affecting the electrode. The work to be described was undertaken with a view to obtaining some clearer conception of the nature of the reduction potentials of bacterial cells, as recorded both by the electrode and by the use of redox indicators. No attempt has been made to study redox equilibria of any particular enzyme system as has been done for example by Lehmann [1929] for the succinate-fumarate system.

EXPERIMENTAL.

The bacteria used in this investigation were *Bact. coli* (Escherich), *Bact. alkaligenes* and *Cl. sporogenes*. Of these, the first is a facultative anaerobe, the second a strict aerobe, whilst the last is a strict anaerobe. They were all grown on a tryptic digest of caseinogen. *Bact. coli* and *Bact. alkaligenes* were grown in Roux bottles for 15–18 hours and *Cl. sporogenes* was grown for 2 days anaerobically. The cultures were centrifuged and washed twice by centrifuging in distilled water, and uniform suspensions were made in water.

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[1] Benn W. Levy Research Student.

Potentials measured at the electrode.

These were measured in an apparatus similar to that described by Green [1933] except that an extra side-tube was added to the reaction vessel by means of which solutions could be added to the fluid in contact with the electrode (Fig. 1). The nitrogen was freed from oxygen by passing over copper treated with palladium at a temperature of 380°. The potentials were measured at room temperature (which was observed at intervals) using a mirror galvanometer in series with which was a resistance of 50,000 ohms to minimise polarisation. Before and after the measurement of the reduction potential, a sample was taken for the determination of the p_H by the quinhydrone electrode. All the measurements of reduction potential are expressed as E_0' at p_H 7.

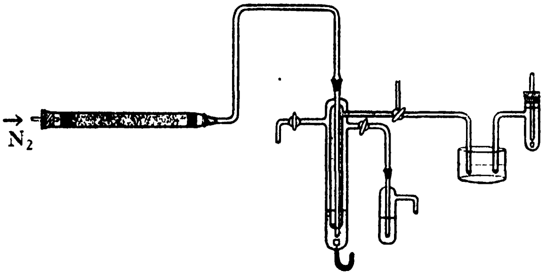

Fig. 1. Apparatus for measurement of reduction potentials.

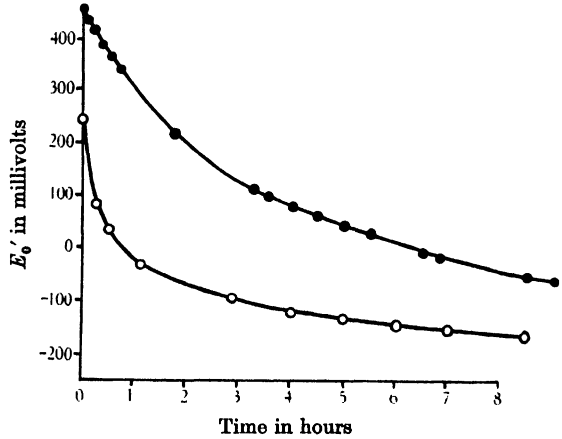

Fig. 2. *Bact. alkaligenes.* Electrode reduction potentials. •—• Fresh suspension. o—o Suspension 2 days old.

The potentials were determined of bacterial suspensions in the presence of an equal volume of $M/20$ phosphate buffer at p_H 7·0, with or without the addition of other substances. In the absence of these additional substances, a slow continuous fall of potential was observed, which continued as long as readings were taken. These in exceptional circumstances were extended over a period of 3 days and no constant value was obtained. The rate of fall of potential was slightly more rapid in the case of *Bact. alkaligenes* than with the other two species, particularly if the suspension had been kept 1 or 2 days in the ice-chest (Fig. 2).

The effect on the potential of adding certain substances was next observed. With *Bact. coli*, the presence of a small amount of $M/10$ sodium succinate solution did not change the rate of fall of potential. With one suspension of *Bact. coli*, a potential of $-0·25$ v. was reached after 10 hours both with and without the addition of succinate. If glucose or sodium formate was added, on the other hand, a rapid fall of potential occurred, which was complete after 15 mins. to 1 hour. Usually this was followed by a gradual rise of about 0·03 v. extending over about 1 hour. The lowest potentials recorded are given in Table I.

Table I. *Reduction potential* (E_0' *at* p_H *7·0 in volts*) *of washed suspensions of* Bact. coli *in the presence of:*

Glucose: $-0·38$, $-0·37$, $-0·35$, $< -0·33$, $-0·36$, $-0·36$, $-0·35$, $-0·37$
Formate: $-0·38$, $-0·40$, $-0·40$, $-0·38$, $-0·38$

The effect of the substrates on the potentials is shown more clearly by following the decrease before and after the substrates are added. This was effected by attaching to the extra side-arm of the reaction vessel a tube containing the substrate solution. It was arranged that the nitrogen passing through the bacterial suspension also passed through this solution, thus ensuring that this was free from oxygen when it was mixed with the bacteria. A typical result is shown in Fig. 3. It will be seen that there is an immediate rapid fall of potential on the addition of glucose. When the minimum potential value has been reached, the addition of formate causes a further slight fall. If formate is added first, the low potential recorded is not influenced by the addition of glucose. This method of observing the potential showed that the addition of succinate has little or no effect on the rate at which the potential falls. Addition of formate after the succinate caused a rapid fall to $-0·38$ to $-0·40$ v. The observed potential of a mixture therefore appears to be that of the most negative system present.

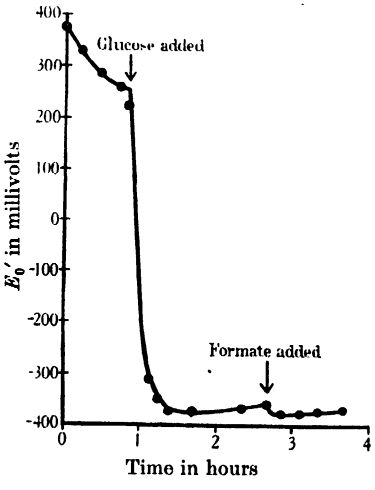

Fig. 3. *Bact. coli.* Electrode reduction potentials.

The effect of toluene on the reduction potential of *Bact. coli* in the presence of glucose was studied in order to see what part glucose dehydrogenase, which is inhibited by toluene, plays in the development of the reduction potential.

Experiment showed that the addition of toluene to the bacteria before the glucose resulted in a very slow fall in potential which, after 24 hours, had only reached 0·0 v. On the other hand, if the toluene was added from the side-tube to the mixture of bacteria and glucose which had been reacting for some time, the rate of fall and the final potential were exactly the same as in the absence of toluene. These results recall those in which methylene blue, although not reduced by toluene-treated bacteria in the presence of glucose, is reduced if the toluene is added after the glucose [Yudkin, 1934, 2]. This was explained as being due to the production of substances from the glucose which, unlike glucose itself, are able to reduce methylene blue in the presence of toluene-treated bacteria. In the case of the reduction potential also it appears that the actual potential is due to the action of the bacteria on the products rather than the initial action of the bacteria on glucose.

The above results were all obtained with *Bact. coli*. With *Bact. alkaligenes* similar results were recorded as far as the absence of effect of succinate is concerned. The effect of glucose is different with the two species of bacteria since the potential of suspensions of *Bact. alkaligenes* is affected comparatively little by this substance. Formate has a more marked effect (Fig. 4). A final value of the potential with this organism was difficult to obtain owing to the continuous, though gradually slowing, fall of potential. At the end of 6 hours, however, the fall is almost immeasurably small and approximate values of the potential for the different substrates can be obtained (Table II).

Table II. *Reduction potentials* (E_0' *at* p_H *7·0 in volts*) *of suspensions of* Bact. alkaligenes *after 6 hours.*

Bacteria alone or with succinate	−0·17
,, +glucose	−0·27
,, +formate	−0·30–0·32

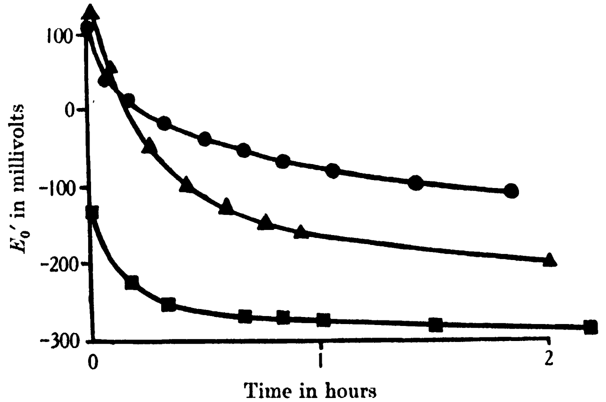

Fig. 4. *Bact. alkaligenes*. Electrode reduction potentials. ●—● Bacteria alone. ▲—▲ Bacteria+glucose. ■—■ Bacteria+formate.

Only a few measurements of reduction potential were made with *Cl. sporogenes*. The fall of potential was very slow indeed, the potential even after 24 hours being only about 0·0 v. The rate of fall and final potential were unaltered by the addition of alanine, which this organism can readily activate as hydrogen donator [Stickland, 1934].

Potentials measured by dye reduction.

In Table III is given a list of the indicators used, together with their potentials at p_H 7·0 for 50 % reduction.

Table III.

	Indicator	E_0' at p_H 7·0 50 % reduction		Indicator	E_0' at p_H 7·0 50 % reduction
1.	Cresolindophenol	+0·210	7.	Janus green	−0·252
2.	Cresyl blue	+0·031	8.	Phenosafranine	−0·256
3.	Methylene blue	+0·011	9.	Rosinduline	−0·281
4.	Indigo carmine	−0·125	10.	Neutral red	−0·340
5.	Nile blue	−0·150	11.	Benzylviologen	−0·359
6.	Cresyl violet	−0·173	12.	Methylviologen	−0·446

Dilute solution of these were placed in Thunberg tubes with the bacterial suspension and phosphate buffer p_H 7·0 and in some cases a solution of substrate. The tubes after evacuation were left at room temperature and observed after about 1 hour.

A suspension of *Bact. coli*, with or without succinate, reduced 1–8 completely, 9 partially and did not affect 10, 11 and 12. With glucose, 1–9 were reduced completely, 10 was doubtful, 11 partially reduced and 12 not affected. With formate, even 12 was slightly reduced. The reduction potentials as recorded by indicators are therefore:

	E_0' in volts
Bact. coli alone or with succinate	−0·27 to −0·30
,, +glucose	−0·32 to −0·37
,, +formate	−0·39 to −0·41

These figures agree fairly well with those given by electrode measurements (p. 1132).

The results with *Bact. alkaligenes* do not show this agreement between the electrical and colorimetric methods. Dyes 1–11 are all reduced by the bacteria alone or in the presence of succinate or glucose (E_0' −0·36 to 0·38). With formate, 12 is also definitely reduced (E_0' about −0·42). Similarly, *Cl. sporogenes* shows potentials by this method which differ greatly from those shown by the electrode. With or without alanine, 1–11 are rapidly reduced at p_H 7·0, indicating a potential of about −0·38 [*cf.* Stickland, 1934].

Potentials measured at the electrode in the presence of indicator.

Series of electrode measurements were made with *Bact. coli* and *Bact. alkaligenes* in the presence of dyes. The results depended on the range of potential of the dye used. If this was very positive, *i.e.* if the dye was rapidly and completely reduced, then the effect was small. For example, if methylene blue was added to *Bact. coli* in the presence of formate, a pause in the falling potential occurred whilst the dye was being reduced (*i.e.* in the region of 0·0 v.) but the subsequent rate of fall and final potential recorded were the same as when no dye was present. Again, if the dye was added from the side-tube after the potential had reached a low value, a slight rise occurred which lasted for a minute or two if the quantity of dye added was small, and then the potential rapidly returned to its previous low value. These results recall those of Cannon *et al.* [1926] which have already been referred to.

If a dye is chosen which is only partially reduced by the system, the potential recorded is approximately that which would be expected from the range of potential of the dye. For example, *Bact. coli* and glucose in the presence of benzylviologen give a reduction potential of −0·36 to −0·38 v. *Bact. alkaligenes* gives with benzylviologen the following reduction potentials measured electrically: alone, or with glucose −0·34 to −0·38 v.; with formate, −0·40 to −0·42 v. These values are those which would be expected from the amount of reduction of the dye used: that is, they agree with the values for the reduction potential obtained colorimetrically. The potentials recorded electrically in the presence and absence of these partially reduced dyes are, therefore, in agreement only in those cases where the electrical and colorimetric values are in agreement. This is shown in Table IV where the values obtained by the three methods outlined above are summarised.

Table IV. *Reduction potentials of bacterial suspensions* E_0' *in volts at* p_H 7·0.

		Potential measured		
Bacteria	Substrate	Colorimetrically	Electrometrically	Electrometrically in presence of partially reduced dye
Bact. coli	None	−0·27 to −0·30	< −0·25	—
	Succinate	−0·27 to −0·30	< −0·25	—
	Glucose	−0·32 to −0·37	−0·36 to −0·38	−0·36 to −0·38
	Formate	−0·39 to −0·41	−0·38 to −0·40	About −0·39
Bact. alkaligenes	None	−0·36 to −0·38	About −0·17	−0·34 to −0·38
	Succinate	−0·36 to −0·38	About −0·17	—
	Glucose	−0·36 to −0·38	About −0·27	−0·34 to −0·38
	Formate	About −0·42	−0·30 to −0·32	−0·40 to −0·42
Cl. sporogenes	None	About −0·38	About 0·0	—
	Alanine	About −0·38	About 0·0	—

The cause of the potential at the electrode.

The main problem was to decide whether the potentials were being caused by contact with the electrode of the bacteria, possibly with adsorbed reactants, or by contact with the electrode of the reactants themselves moving freely in the solution. A series of experiments to test this point was carried out with *Bact. coli*.

A bacteria-free filtrate of the mixture after the bacteria had acted on glucose for a long time was found to give a potential of about −0·20 v. which was almost certainly due to the glucose itself. However, it was found possible to obtain the low potentials recorded above in bacteria-free solutions by use of a thin collodion sac.

A small beaker with a well-fitting rubber stopper was used as reaction vessel. In the stopper were inserted two platinum electrodes, a tube through which the nitrogen bubbled into the solution, an exit tube for the nitrogen and a KCl-agar bridge making contact with the KCl half-element. Round one electrode was tied a collodion sac containing a mixture of buffer and substrate solution. In the beaker was placed the mixture containing bacterial suspension, buffer and substrate solution. Thus the electrode within the sac was in contact only with substances capable of diffusing through the sac.

It was found that if the sac was thin and not too wide, the final potentials recorded by the covered electrode were, within a few millivolts, the same as those recorded by the outside electrode. In one experiment with glucose for example a potential of −0·330 v. was recorded by the outside electrode and −0·327 v. by the covered electrode. Similar results were obtained with formate. It was necessary to leave the experiment for 6–8 hours before this agreement was obtained.

At the end of each experiment, the apparatus was dismantled carefully in order to observe whether the bacteria had passed into the sac through any small hole which had developed. In certain cases where this did occur it was very obvious, the liquid in the sac being quite milky and rapidly dripping out of the sac even when no visible hole existed.

DISCUSSION.

It seems fairly certain from the results given that the reduction potential of a bacterial suspension, as measured at an electrode, is produced by substances in solution in the suspension. The potentials recorded for suspensions in the absence of added substrate are probably due to substances exuding from the bacterial cells. This is supported by two observations; firstly, by the very slow and prolonged fall of potential, and secondly by the fact that older suspensions (of *Bact. alkaligenes*), in which presumably more of these substances have diffused out, show a more rapid fall of potential.

Of the substrates examined, two, namely glucose and formate, produce a potential more negative than the bacterial suspension alone. It may be inferred that the substances produced from other substrates, such as succinate, either have no effect at the electrode or have much less effect than the substances produced in the bacterial suspension itself. The equilibrium potential of the succinate-fumarate system is given as 0·00 v. at p_H 7·0 and 30° [Lehmann, 1929].

A possible explanation of the low potentials recorded in the presence of glucose and formate is that hydrogen is being produced, giving a partial pressure of $1-10^{-2}$ atmospheres. If this is correct then the reduction potential would be expected to depend on the rate at which the nitrogen was passed into the vessel since this would alter the amount of hydrogen left in the solution. This was found not to be the case. Also, a suspension capable of producing more hydrogen (*i.e.* containing a more active hydrogenlyase) might be expected to produce a more negative potential. The suspensions of *Bact. coli* as grown for this experiment contain no formic hydrogenlyase and very little glucose hydrogenlyase [Yudkin, 1932]. As has already been suggested [Yudkin, 1934, 1], it is conceivable that very small amounts of hydrogen are actually produced from formate in such suspensions but in quantities insufficient to be measured manometrically. These very small quantities might, however, be sufficient to affect the electrode. Suspensions of *Bact. coli* grown on glucose broth and hence containing active formic and glucose hydrogenlyases were prepared. The potentials of these suspensions in the presence of either glucose or formate did not, however, produce a more negative reduction potential than the other preparations.

The argument against hydrogen as the cause of the low potentials with glucose and formate in the case of *Bact. alkaligenes* is still stronger since in no circumstances is it possible to obtain measurable quantities of hydrogen by the action of this species on either glucose or formate.

We can also deduce from the experimental results that the substances responsible for the low potentials do not do so by virtue of being hydrogen donators in the presence of the bacteria. One might imagine that the activation of a substrate as a hydrogen donator involves a "loosening" of a hydrogen atom and that it is this which affects the electrode. But the results obtained with *Cl. sporogenes* in the presence of alanine show that this is not so.

We must, therefore, conclude that the potentials recorded by an electrode immersed in a bacterial suspension with or without added substrates are due to unknown substances either coming out of the bacterial cells or being produced from certain of the substrates (*e.g.* glucose and formate). That these substances affect the electrode at all and so give a measurable potential by no means indicates that a definite oxidation-reduction equilibrium is being measured. In fact, the slow and continuous fall of potential in the absence of glucose or formate and the ease with which all the systems are polarised suggest that the effect on the electrode of some of these substances at any rate is quite fortuitous and that the values obtained for the potential by electrical measurement gives very little knowledge of the state of the cells.

The above discussion was confined entirely to potentials measured at the electrode in the absence of dyes. We have seen that colorimetric measurements of the reduction potentials often give results differing widely from those obtained by such electrical measurements, although they agree with electrical measurements made in the presence of partially reduced dye. The differences may be explained along the following lines. It has been pointed out by Green *et al.* [1934] that the reduction of dyes by bacterial cells depends on the existence in the cells of positive (oxidising) and negative (reducing) systems. Whether or not a certain dye will be reduced does not depend on the actual potential of the cell. A small quantity of redox indicator will be completely oxidised, completely reduced or partially reduced depending on the relative rates of oxidation and reduction of the particular dye by the positive and negative systems. In the case of a dye which is partly reduced, we may perhaps speak of "reduction equilibrium" of the dye in question, where the rate of reduction of the dye by the negative systems is equal to the rate of its oxidation by the positive systems. In the presence of partially reduced dye, an electrode will naturally take up the potential value of this mixture of oxidised and reduced dye since both forms are active at the electrode. In other words, colorimetric measurements of the reduction potential will agree with electrode measurements in the presence of partially reduced indicator, and both are measuring the "reduction equilibrium" of the dye.

On the other hand, as shown above, the potentials recorded at the electrode in the absence of dye are due to the existence in the mixture of certain diffusible substances which are able to affect the electrode. These are quite fortuitous and have nothing to do with the oxidising and reducing mechanisms of the cells.

There is, therefore, no method by which the reduction potential of a cell, in the sense commonly used, may be measured. The author is of the opinion that the conception of the reduction potential of the living cell as an equilibrium exhibited by the whole cell has no great meaning. It appears much more likely that different parts of the cell in which the various systems are differently proportioned will have very different reducing intensities. The use of the term reduction potential, if justified at all at present in biology, must be restricted to reversible systems in true equilibrium such as reduced and oxidised cytochrome or succinic dehydrogenase in the presence of fumarate and succinate.

SUMMARY.

1. The reduction potentials of suspensions of *Bact. coli* and *Bact. alkaligenes* were measured (1) electrometrically, (2) colorimetrically by redox indicators, and (3) electrometrically in the presence of indicators. A few measurements were also made with *Cl. sporogenes*.

2. Both *Bact. coli* and *Bact. alkaligenes*, either alone or in the presence of succinate, show a slow continuous fall of potential at the electrode, which never

reaches a constant value. Glucose and formate induce more negative potentials, which in the case of *Bact. coli* soon reach minimum values.

3. These potentials have been shown to be due to electromotively active molecules capable of passing through a collodion sac and not to the bacteria themselves or bacteria *plus* adsorbed substances.

4. The reduction potentials recorded by indicators, although sometimes in fair agreement with the electrode values (*Bact. coli*), often bear no relation to the latter (*Bact. alkaligenes* and *Cl. sporogenes*). The electrode measurements in the presence of appropriate dyes give values agreeing with those indicated by the colorimetric study.

5. It is pointed out that the methods involving the use of these redox indicators merely measure the "reduction equilibrium" of the dyes used, that is, the point at which the rates of oxidation and reduction of the dye are the same. On the other hand, measurements of the electrode potential without an appropriate indicator give no information beyond the fact that there are certain (diffusible) substances in the solution which can affect the electrode. We have, in fact, no method whereby the true reduction potential of cells can be measured.

6. There is no correlation between the mode of life of the organism and the "reduction potential" measured by these methods. *Bact. coli* is a facultative anaerobe, *Bact. alkaligenes* a strict aerobe and *Cl. sporogenes* a strict anaerobe, but the reduction potentials show no correlative differences.

I desire to thank both Prof. E. K. Rideal for suggesting this investigation and Dr D. E. Green for helpful discussion during the course of this work.

REFERENCES.

Aubel and Aubertin (1927). *Compt. Rend. Soc. Biol.* **97**, 1729.
—— —— and Mauriac (1928). *Compt. Rend. Soc. Biol.* **98**, 589.
—— and Genevois (1927). *Compt. Rend. Acad. Sci.* **184**, 1676.
Cannon, Cohen and Clark (1926). *U.S. Public Health Reports*, 1926. Supplement 55.
Gillespie (1920). *Soil Science*, **9**, 199.
Green (1933). *Biochem. J.* **27**, 678.
—— Stickland and Tarr (1934). *Biochem. J.* **28**, 1812.
Hewitt (1930, 1). *Biochem. J.* **24**, 512.
—— (1930, 2). *Biochem. J.* **24**, 669.
—— (1930, 3). *Biochem. J.* **24**, 670.
Lehmann (1929). *Skand. Arch. Physiol.* **58**, 173.
Stickland (1934). *Biochem. J.* **28**, 1746.
Yudkin (1932). *Biochem. J.* **26**, 1859.
—— (1934, 1). *Biochem. J.* **28**, 1454.
—— (1934, 2). *Biochem. J.* **28**, 1463.

CXLIII. THE DISTRIBUTION OF STEROLS IN HUMAN FAECES.

AN EXAMINATION OF ILEAL CONTENTS.

By JOHN ADDYMAN GARDNER, HUGH GAINSBOROUGH
AND RUTH MONA MURRAY.

From the Biochemical Department, St George's Hospital.

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Long ago Flint [1862; 1868] observed that cholesterol undergoes a modification in the human intestine, and that it is found in the faeces as "stercorine." This substance, rediscovered by Bondzynski [1896] who gave it the name of "coprosterine," was more fully described by Bondzynski and v. Humnicki [1896]. The formula $C_{27}H_{48}O$ was ascribed to it and confirmed by the analysis of derivatives, and its discoverers regarded it as a dihydrocholesterol formed by bacterial reduction in the intestine. Support was lent to this hypothesis by the observation of Müller [1900] that in a patient kept for a long time on a milk diet, which presumably altered the intestinal flora, the sterol of the faeces was excreted as unchanged cholesterol. Later, Gardner [1921] isolated from human faeces, in addition to coprosterol, small quantities of β-cholestanol and cholesterol. These substances were found not only in the mixed faeces of a village community but also in the excreta of soldiers in training. Recently, Schoenheimer [1934] has perfected an improved method suitable for small quantities of material for the isolation of cholesterol from mixtures of β-cholestanol and coprosterol and has confirmed the presence of cholesterol in human faeces.

Fox and Gardner [1925] published the result of a long series of experiments on the cholesterol metabolism of breast-fed infants and found that the crystalline sterol of the faeces consisted only of cholesterol. At what stage of life the change of the faecal sterols, from the cholesterol of infant's faeces to the mixture of coprosterol, β-cholestanol and cholesterol characteristic of adult human faeces, takes place has yet to be determined.

Some evidence has accumulated as to the origin of the different forms of faecal sterol. The observation of Boehm [1911] that an isolated loop of intestine, accidentally produced in a woman under operation and left *in situ* for years, contained sterile material, the sterol content of which was solely β-cholestanol, suggests a secretion of this sterol through the wall of the intestine into its lumen. Beumer and Hepner [1929] found cholesterol in the colonic contents of a bile fistula dog fed on a lipoid-free diet and concluded that cholesterol was secreted by the large intestine. Schoenheimer and v. Behring [1930] examined the secretions of the isolated large intestines of a series of dogs. After suitable washing out of the secretions and sealing of the intestinal tube for a length of time, he finally obtained a cheesy material, similar to that obtained by Boehm, containing sterol of which 52 % was saturated, and showed it to be mainly β-cholestanol, the remainder being cholesterol. Sperry [1926–27] fed dogs which had had a bile fistula made, on a lipoid-free diet, and the unsaponifiable matter obtained from the faeces was afterwards closely examined by Schoenheimer and Sperry [1934]. They concluded that, despite a sterol-free diet and the total exclusion of bile from the intestine, coprosterol was the principal sterol excreted by their dogs: only 11 % of the total sterols excreted was unhydrogenated (probably cholesterol); β-cholestanol was also present. They considered that in such dogs coprosterol must be formed from cholesterol which is secreted into the intestine through the intestinal wall. It is to be noted that in Boehm's and Schoenheimer's experiments, where β-cholestanol appeared to be the predominant sterol, the intestinal contents were sterile.

The question arose as to how and where the faecal coprosterol was produced. It is to be remembered that coprosterol is not of the same stereoisomeric series as cholesterol, the direct hydrogenation product of which is β-cholestanol. So far the only explanation offered is that coprosterol is produced from cholesterol by bacterial action, and the only evidence available here is that of Müller mentioned above and that of Dam [1934] who thought he could show an increased formation of coprosterol in faecal putrefaction experiments in which cholesterol had been added, though the production of coprosterol here was only inferred from the solubility curve of the digitonide and the increase measured by Schoenheimer's method, which is possibly scarcely accurate enough to permit such conclusions to be drawn.

We feel by no means certain that the bacterial hydrogenation of cholesterol to produce coprosterol, if true, is the whole story. We have been amazed at the rapidity with which large quantities of coprosterol can be produced under certain circumstances. Dorée and Gardner [1908] made an extensive series of feeding experiments on a trained dog, using a number of different diets for long periods. It has been necessary to reinterpret the findings in that paper in the light of the greater knowledge of to-day, and we can safely conclude that the sterol matter isolated from the dog's faeces when the animal was fed on diets containing cholesterol in what might be considered normal amounts consisted of a mixture of cholesterol and β-cholestanol. Coprosterol was not isolated. However, when this same animal was fed on a diet of cooked sheep's brains, it excreted quantities of coprosterol. The actual quantity isolated in apparently pure condition was 1·2 g. per day, whereas approximately only 1 % of the total sterols isolated consisted of a mixture of cholesterol and β-cholestanol. Similar experiments by Ellis and Gardner [1909] with cats produced similar results, except that the cat, on a normal diet, produced coprosterol more easily than the dog. The contrast is very marked in the case of the herbivora [Dorée and Gardner, 1908] with which in no case could any trace of animal sterol be found in the faeces. It is curious that the normally fed dog should excrete cholesterol and β-cholestanol but no isolable coprosterol, and yet when fed on sheep's brains a mechanism should be available, of which there was no previous sign, which could produce such large quantities of coprosterol. If the mechanism is bacterial then why is there so little evidence of coprosterol formation with an ordinary diet? It is conceivable that on the brain diet a different intestinal flora appeared, but this explanation does not seem very likely, because the dog after feeding on sheep's brains showed a return to normal faecal sterol distribution on the withdrawal of that diet. In other words, if the reduction of cholesterol to coprosterol can be so extremely efficient in the brain-fed animal, why should the normally fed dog excrete so much cholesterol and β-cholestanol? At the same time (1908) it was shown that the total sterol excreted was much less than the sum of the total intake *plus* the biliary cholesterol, whence it was inferred that, as it seemed unlikely that cholesterol was destroyed in the gut, cholesterol was being absorbed in the alimentary canal. This has been confirmed, though coprosterol and β-cholestanol have been shown by Schoenheimer *et al.* [1930] not to be so absorbed from the intestine. We suggest that the mechanism of coprosterol formation shown by the dog and the cat protects the animals from the effects of over-absorption of cholesterol which can be so readily observed in the case of rabbits, where cholesterol is avidly taken up from the intestine, eventually producing a lipoid infiltration of the organs and tissues.

The question as to the site of coprosterol formation in man seemed to us easy of solution, but there have been many difficulties in obtaining uncontaminated ileal faeces from surgical cases. However, the opportunity finally presented itself of examining two cases in which ileostomy had been performed. In 1930 we had analysed some caecostomy faeces from a case of ulcerative colitis and obtained a clear-cut result, but as the faeces had already entered the colon we did not consider the unsupported result sufficiently significant. In this case 0·1 g. of crystalline material was obtained directly by crystallisation from the unsaponified matter of the ether extract and a further 0·12 g. after treating the residues with digitonin and subsequently splitting the digitonide in the usual way. The former portion melted at 146–147°, had $[\alpha]_D^{20°}$ −40·7° and was clearly cholesterol; the portion obtained by digitonin had melting point 142–143°, and $[\alpha]_D^{20°}$ −31·6° and was found to contain about 10 % of saturated sterol, probably β-cholestanol. No trace of coprosterol was isolated.

The ileal contents examined were from the following two cases:

M. C. Female, aet. 32. Ulcerative colitis. A caecostomy had been performed in August 1933 and, as only temporary benefit was obtained, a transverse ileostomy was made in July 1934 about 6 in. from the ileal termination.

L. H. Female, aet. 30. Ulcerative colitis. A caecostomy had been performed in May 1928; this was remade in November 1930, but as symptoms recurred later a transverse ileostomy was made in November 1933. This case unfortunately had a small ileo-caecal fistula, but it is unlikely that caecal contents could have flowed back into the ileum, hence the ileostomy discharge was considered to be uncontaminated ileal contents.

In both cases no grease of any kind was used to protect the skin of the abdomen: a kaolin paste was used for this purpose and great care was taken that no caecostomy discharges were included in the ileal excreta.

Exp. 1. Ileal faeces from M. C. were collected during the whole of May and June 1934. These were dried partly *in vacuo*, partly by heat, and as it was difficult to get rid of all the moisture the faeces were soaked in alcohol first cold, then warm. The alcoholic extracts were evaporated to dryness and the residue extracted with ether. The dried faeces were then exhaustively extracted with ether in a special Soxhlet apparatus without rubber connections, corks or taps. The two ether extracts were put together and made up to known volume. The solution was dark green and absorption bands indicated that this was due to chlorophyll. This extract after suitable concentration was hydrolysed with sodium ethoxide in alcoholic solution, the unsaponifiable matter extracted in the usual manner and this material crystallised from methyl alcohol. Successive crops of crystalline material, which very closely resembled cholesterol, were separated and 4·63 g. were obtained. The residual oil obtained from the mother liquors weighed 1·03 g.; this was precipitated by digitonin in the usual manner, and 0·13 g. of cream-coloured crystals was obtained by splitting the digitonide. The total crude sterol was altogether 4·76 g. This was brominated by the Windaus method and 4·67 g. of the dibromide were obtained, M.P. 112–113°. This was debrominated with zinc dust and acetic acid and after repeated crystallisation 1·77 g. of typical cholesterol crystals were obtained; M.P. 144–145°. This was again brominated, yielding a dibromide of M.P. 113–114°, and finally a sample of cholesterol was obtained (0·35 g.), of M.P. 146–147° and $[\alpha]_D^{19°}$ −36·8°. Further examination was made of the second crops of dibromide that were precipitated by water in both brominations. These substances after debromination were converted into the acetates and crystalline substances obtained, M.P. 117–118°, which on recrystallisation yielded pure cholesteryl acetate. Clearly some phytosterol was present but in quantity insufficient for isolation by the method suggested by Windaus [1906]. The mother-liquors were similarly examined with identical results.

Thus the great bulk of the original crude sterol consisted of cholesterol, but after much search only traces of phytosterol were detected and no β-cholestanol or coprosterol could be isolated.

Exp. 2. 115 g. of dried ileal faeces were obtained from L. H. These were extracted with ether, and the total ether extract consisted of a brown oil weighing 48 g. This, after saponification, yielded 40 g. of unsaponifiable material and it was then discovered that instructions in the ward had been disregarded and the patient had been given paraffinum liquidum by mouth. A digitonin estimation on an aliquot part of the ether extract showed that it contained 0·796 g. sterol in the whole specimen. The unsaponifiable matter was then dissolved in alcohol, precipitated with digitonin, left overnight, the separated digitonide filtered off and washed free from "paraffin" with ether. The digitonide so obtained was decomposed by the pyridine-ether method and the recovered sterol crystallised from methyl alcohol in faintly pink plates. The first crop consisted of 0·6 g. cholesterol, M.P. 144–145°, and this, recrystallised, yielded 0·46 g. of colourless crystalline cholesterol, M.P. 146–147°, with $[\alpha]_D^{18^\circ} -37{\cdot}7^\circ$. Further portions from this recrystallisation gave material with M.P. 143–144°. The second crop (0·07 g.), the third crop consisting of brownish crystals in oil, some further material obtained from some digitonide which had been finally separated from "paraffin" with great difficulty and all the residues from the first crop were mixed together, weighing 0·22 g. in all. This was brominated by Schoenheimer's method, the dibromide filtered off, digitonin in alcohol added and after 18 hours 0·028 g. of digitonide was deposited, and the filtrate yielded after a further 24 hours 0·036 g. of digitonide. These were separately decomposed by pyridine and ether, the first yielding 0·007 g., and the second 0·005 g. These were added together and crystallised from methyl alcohol, yielding plates which gave a strong Burchardt-Liebermann reaction. A solution of the whole material in chloroform was compared by means of this reaction with standard solutions of cholesterol and it was found that over 75 % of the material was cholesterol.

Evidently the bulk of the material in this case was cholesterol and no β-cholestanol or coprosterol could actually be isolated, though it was very likely that some traces of β-cholestanol were present.

Exp. 3. It was thought wise to repeat the analysis of further material from M. C., taking strict precautions as regards eliminating paraffin administration, and eliminating green vegetables and fruit from the diet in order to reduce the intake of phytosterols as far as possible. ·620 g. of dried ileal excreta, collected during November and December 1934, were thoroughly extracted with ether and 36·2 g. of ether-soluble matter obtained. The total sterol in this was estimated by digitonin in an aliquot part and amounted to 7·08 g. After saponification of the ether extract 6·6 g. of unsaponifiable matter were obtained and this was recrystallised from methyl alcohol. Crop I (5·015 g.) was further purified and crop I′ (3·22 g.) of pure cholesterol was thus obtained with M.P. 147°, $[\alpha]_D^{20^\circ} -35{\cdot}3^\circ$, and from the mother-liquors of this 1·29 g. sterol were obtained, M.P. 139–140°, $[\alpha]_D^{20^\circ} -37{\cdot}6^\circ$. The pure sample (crop I′) was brominated by the Windaus method and yielded an initial precipitate (A) of dibromide (3·8 g.), M.P. 112–113°, and a further crop (B), by adding water (1·25 g.). From A, after reduction, 1·6 g. of pure cholesterol were finally obtained, M.P. 148°, clearing at 150°, and the material from the mother-liquors was evaporated to dryness and benzoylated by the pyridine method, yielding a solid melting at 147°, clearing at 173°; a further sample from the mother-liquors melted at 146° and cleared at 168°. Neither of these benzoates on melting and cooling showed any play of colours. The material B yielded no crystalline matter after reduction and it was therefore all benzoylated as before and a sterol benzoate obtained melting at 146° and clearing at 171° without any play of colours. Crop II from the crystallisation of the crude unsaponifiable matter consisted of 0·65 g. of orange stained crystals. From this 0·49 g. of a benzoate was obtained melting at 141° and clearing at 167° without any blue flash but on cooling there was a quick play of colours. This effect was matched by a sample of cholesteryl benzoate to which 5 % of β-cholestanyl benzoate had been added. Small additional crops from this crystallisation showed the same behaviour. Crop III from the unsaponifiable matter consisted of 0·65 g. of orange crystals mixed with oily matter. Some of this was unfortunately lost but 0·15 g. of a benzoate was obtained, M.P. 129–133°, clearing at 168°, showing similar colour changes to the material from crop II but weaker.

This experiment amply confirms the previous findings that only cholesterol can be isolated but that traces of β-cholestanol can be detected.

It may be noted here that cholesteryl benzoate made from pure cholesterol does not show any trace of play of colours on melting and cooling, whereas addition of β-cholestanol benzoate to cholesterol benzoate in increasing proportions produces such phenomena on melting. This can be used for a rough indication of the amount of β-cholestanol in a sample of cholesterol by comparison with artificial standard mixtures.

It had occurred to us some years ago that the sterol distribution in human faeces might be affected by the presence or absence of bile. We collected the clay-coloured faeces of two cases of complete biliary obstruction and recovered the sterols therefrom. The analyses were not carried out to the ultimate fractions but it became clear that the bulk of the faecal sterols in these cases consisted of coprosterol as in normal subjects.

DISCUSSION.

It is quite clear from these three analyses of ileostomy excreta and one of caecostomy excreta that the sterol content is quite different from that of normal human faeces. From the former only cholesterol can be isolated and traces of β-cholestanol detected, whereas the latter contain sterol mainly as coprosterol, smaller quantities of β-cholestanol and traces of cholesterol. It follows that the production of coprosterol must occur in the colon. The earlier experiments of Dorée and Gardner showed that the dog on a normal diet excreted mainly cholesterol in its faeces and the cat cholesterol and coprosterol. We realise now that as the apparently pure cholesterol isolated in these cases yielded benzoates which showed marked colour changes on melting there must have also been some β-cholestanol present. The mechanism of coprosterol formation was easily brought into evidence by feeding the animals on a diet of sheep's brains, and it is difficult to understand how such an action can be due solely to bacterial hydrogenation for, if this were the case, there is no obvious reason why this action should remain latent in normally fed animals, in which little or no coprosterol is formed.

SUMMARY.

1. Coprosterol could not be isolated from intestinal contents discharged from either a terminal ileostomy or a caecostomy.

2. It follows that coprosterol formation must occur in the colon.

3. We think that the hypothesis that coprosterol formation results from the bacterial hydrogenation of cholesterol is insufficient.

We wish to express our thanks to our colleagues at St George's Hospital for allowing us access to these cases.

REFERENCES.

Beumer and Hepner (1929). *Z. ges. exp. Med.* **64**, 787.
Boehm (1911). *Biochem. Z.* **33**, 474.
Bondzynski (1896). *Ber. deutsch. chem. Ges.* **29**, 476.
—— and v. Humnicki (1896). *Z. physiol. Chem.* **22**, 396.
Dam (1934). *Biochem. J.* **28**, 815, 820.
Dorée and Gardner (1908). *Proc. Roy. Soc. Lond.* B **80**, 212, 228.
Ellis and Gardner (1909). *Proc. Roy. Soc. Lond.* B **81**, 505.
Flint (1862). *Amer. J. Med. Sci.* 44.
—— (1868). Recherches expérimentales sur une nouvelle Fonction du Foie (Paris).
Fox and Gardner (1925). *Proc. Roy. Soc. Lond.* B **98**, 90.
Gardner (1921). *Biochem. J.* **15**, 251, 261.
Müller (1900). *Z. physiol. Chem.* **29**, 129.
Schoenheimer (1934). *J. Biol. Chem.* **104**, 355.
—— and v. Behring (1930). *Z. physiol. Chem.* **192**, 77, 97, 102.
—— —— Hummel and Schindel (1930). *Z. physiol. Chem.* **192**, 73.
—— and Sperry (1934). *J. Biol. Chem.* **107**, 1.
Sperry (1926–27). *J. Biol. Chem.* **71**, 351.
Windaus (1906). *Chem. Ztg.* **30**, 1011.

Note. In our last paper, *Biochem. J.* (1934), **28**, 1635, we regret that we inadvertently stated that Schoenheimer had found up to 10 % of β-cholestanol in gall stone cholesterol. This figure should have been 1·0 %. We thank Dr Schoenheimer for drawing our attention to this error.

CXLIV. A STUDY OF THE ANTENATAL SECRETION OF THE HUMAN MAMMARY GLAND AND A COMPARISON BETWEEN THIS AND THE SECRETION OBTAINED DIRECTLY AFTER BIRTH.

By SIBYL TAITE WIDDOWS, MARGARET FRANCES LOWENFELD, MURIEL BOND, CECILIA SHISKIN and EFFIE ISABEL TAYLOR.

From the London (Royal Free Hospital) School of Medicine for Women.

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In a fairly complete study already made by us on the composition of human milk obtained directly after parturition and during the later stages of lactation [Lowenfeld *et al.*, 1927; Widdows *et al.*, 1930; Widdows and Lowenfeld, 1933], certain facts of a rather unexpected nature with regard to its composition in the early stages after labour were reported.

It has been generally recognised that the secretory activity of the maternal mammary gland commences before the expulsion of the foetus. An opportunity was given us for the study of samples of antenatal secretions, and since as far as we could ascertain very little work had been done on this subject, it was decided to undertake an investigation, the results of which are recorded in the present paper.

Work has been done on the antenatal secretion of cows, and it should be emphasised that workers on this line have had the very great advantage of being able to study in the same animal the progressive effects of pregnancy on the composition of the secretion. In particular, the work of Houdet [1894], and later Eckles and Palmer [1916], Woodman and Hammond [1922; 1923], and Asdell [1925] is interesting in view of certain results obtained by us.

Woodman and Hammond, working with heifers, analysed the secretion at 15, 22 and 28 weeks' gestation, the secretion between these stages being removed at intervals but not analysed, while Asdell removed and analysed the secretion at intervals from the 4th week of gestation up to parturition (total number of weeks of gestation being 41), first taking samples biweekly and afterwards weekly. Woodman, Hammond and Asdell all arrived at very much the same conclusion.

Up to the 15th week they obtained a serous secretion containing all the constituents found in milk, but with only traces of fat, and with small amounts of lactose, caseinogen, globulin and albumin, the globulin predominating over the caseinogen and albumin. About halfway through pregnancy the character of the secretion changed and it became thick, viscid and honey-like, with a high content of solid, chiefly protein. The protein consisted almost entirely of globulin, no caseinogen at all being found by Woodman and Hammond, and only a very small amount by Asdell. Traces of lactose were present and a very small amount of fat, and, in fact, Woodman and Hammond looked upon the secretion as a very concentrated solution of globulin. At about the 28th to the 30th week this viscid secretion gave place to a milk-like fluid in relatively large quantity which contained appreciable percentages of the normal constituents of colostrum. When the cow is milked up to parturition, as shown by Asdell, the colostrum milk closely follows the composition of the prepartum fluid which has been approximating to true milk.

If the viscid secretion is not removed, then the transition to the colostral phase appears to be more gradual, and it is only at the time of parturition that the secretion assumes the character of normal colostrum with a high percentage of globulin.

These workers concluded that during the early months of pregnancy the secretory activity of the gland is present but is feeble. At about halfway through pregnancy, which appears to correspond to a definite stage in the development of the gland, the normal activity of the cells, which results in the production of the caseinogen, lactose and fat, seems to be inhibited. If the viscid secretion at this stage is removed, the normal activity of the gland is gradually resumed in the same way as frequent milking stimulates activity during normal lactation.

Outside the limits of normal lactation, globulin seems a characteristic constituent of the mammary secretion, the protein of the secretion at a certain stage consisting practically entirely of globulin.

A tentative suggestion is put forward by Asdell, as a result of this study, that the globulin is present in the mammary ducts as a result of the change from growth to secretion on the part of the mammary cells. If the mammary secretion is removed at this stage, and continually removed as pregnancy proceeds, the proportion of globulin rapidly decreases, while if the secretion is allowed to remain in the gland, the globulin percentage remains high up to the time of parturition, and in the colostrum milk immediately after it, and only acquires the percentage found in ordinary milk as the result of continuous removal of the milk after parturition.

Eccles and Palmer in their work on cows, concluded that when cows are milked up to the time of parturition, the colostrum milk follows closely the composition of the milk given before birth. As the cow approached parturition, these workers found a marked increment of the heat-coagulable proteins which reached their maximum in the first milk obtained after birth. They concluded from these results that the high content of heat-coagulable proteins was the chief, if not the only real effect of parturition on the milk. This does not seem to accord with Asdell's later work, and although these workers only milked continuously for a month before birth, and they were dealing with cows and not heifers, one would have expected a month's continuous milking to have reduced and not increased the heat coagulable-proteins. The secretion during labour was not investigated by these workers. In our work such a complete investigation as the above was not possible. The secretion investigated was that which could be obtained (*a*) once or twice from the same woman during the 2 months before parturition, but (*b*) more often single scanty samples obtained from different women during this period. In view of the results of Woodman, Hammond and Asdell, on cows, it was thought that although the period of study of the antenatal secretion would be more limited in our case, such an investigation would be interesting. It was hoped that similar and comparative results would be obtained for the last 2 months of pregnancy, so that what was experimentally proved in the case of cows for the very early secretion might be inferred to be true of women, where the difficulties of obtaining this secretion make experimental work on such samples impossible.

In this paper it will be seen that to a certain extent our results are comparable, but that there are certain points not in accord and which at present lack explanation.

Czerny and Keller [1925] point out that for the analysis of the secretions of the mammary gland before parturition, there are only the incomplete analyses of Clemm [1845], whose figures are referred to by Engel [1911][1]. The figures obtained from the table in Engel's paper are the results of six analyses from the secretion of one woman. The figures obtained from such a method of investigation taken in conjunction with the probably less accurate methods of investigation available at this early date afford little material for comparison.

METHODS AND OBSERVATIONS.

The material for our work has been obtained from cases both primiparae and multiparae in the Maternity Department of the St Pancras Hospital and from the Shoreditch Carnegie Welfare Settlement.

In only a few cases has it been possible to deal with the same woman throughout the antenatal course, but the secretions from a large number of different cases have been studied, extending over a period from 2 months to the day of delivery. It is felt that to a large extent individual differences may be eliminated in this way and some generalisations may be drawn.

Cases investigated—total number 53.

1. Cases where specimens were obtained only before labour:
   (*a*) 26 cases where a single specimen was obtained;
   (*b*) one case where two specimens were obtained.
2. Cases where specimens were obtained both before and after labour:
   (*a*) 16 cases (including 4 cases in labour) from whom a single specimen was obtained before labour;
   (*b*) 10 cases from whom more than one specimen was obtained before labour, giving in all 34 specimens distributed as follows:

6 cases gave 2 specimens
1 case gave 3 ,,
1 ,, 4 ,,
1 ,, 5 ,,
1 ,, 10 ,,

3. These cases gave a total number of 78 specimens examined before labour.

The chemical constituents estimated were protein, ash, sugar, calcium, phosphorus and chlorine.

The methods employed in the estimations were those recorded in our earlier papers on milk [Lowenfeld *et al.*, 1927; Widdows *et al.*, 1930].

A. *Physical properties of the secretions studied.*

An examination of the mammary secretion before and after labour shows marked differences in its physical properties.

1. *Volume.* In the case of multiparae, after the first day after parturition the volume of the fluid obtained was found to vary from 10 to 30 ml., and in one woman (J.) investigated on the second day the volume obtained was as much as 90 ml. In the case of primiparae, the volumes were found to vary considerably, and sometimes for the first few days after birth were noticeably small.

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[1] So far it has not been possible to obtain a paper by Guiraud [1897] in which all the early work on colostrum up to 1897 is collected including the figures of Clemm.

With very few exceptions, large volumes were not found to be obtainable before labour. Of the 64 women investigated, 9 specimens had a volume under 2 ml., 39 between 2 and 3 ml., 10 between 4 and 6 ml., and 6 between 6·5 and 7·5 ml. Occasionally an exception occurred—for instance, a multipara (H.) gave a volume of 22·5 ml. 2 weeks before labour, and another, a primipara (Ha.) gave, 5 weeks before labour, a volume of 38 ml. In two other cases a volume of 11 ml. has been obtained.

This great difference with regard to volume naturally suggests that the early secretion may merely be a concentrated form of later milk, and upon the elucidation of this point the interest of an investigation of the antenatal secretion depends. If the relative values of the different constituents of the gland are found to be approximately similar on both sides of the line of labour, then we are dealing with a physical change only, and the type of secretory activity is identical before and after labour.

A glance at the tables given below for sugar and calcium, compared with those for protein and ash, shows immediately that this is not the case. Each of the calcium and the sugar curves from 30 days before to 10 days after labour shows a fairly steep rise, settling down later to a more or less even value. In contrast with these curves, the protein values of the antenatal secretions never fall below 2·69 %, with an average of 8·9 %, and from these high values the curve declines steadily during the 10 days after parturition to 1·5 %, above which its value never rises (as shown in our paper on later milk [Widdows *et al.*, 1930]). It is clear therefore that the differences that occur between the fluid obtained from the mammary gland before and after labour must be due to a difference in the secretory activity of the gland and not merely to the variation of the water content in an otherwise standard fluid. For instance, one example (F.), 4 days after parturition gave a protein value of 1·62 % and at the same time 0·042 % CaO, 6·43 % sugar, and 0·28 % ash. The antenatal secretion 9 days before birth gave a protein value of 9·14 %, which on the assumption that there is merely a variation in the water content of the secretion should have given for CaO 0·238 %, for sugar 36·27 %, and for ash 1·57 %, whereas the percentages actually found were 0·0443 % CaO, 4·72 % sugar, and 0·56 % ash.

In view of the results obtained by Woodman, Hammond and Asdell on heifers and cows, it is possible that the explanation lies in the fact that the fluid before parturition in women is not removed from the breast.

2. *Colour and viscosity.* It is well known that the general physical properties of colostrum and early milk are different from those of later milk, and attention has been drawn to this point in our paper on early milk [Lowenfeld *et al.*, 1927]. The antenatal secretion seems to fall into three classes:

(1) A beige-coloured watery secretion, with fat globules sometimes separate from the watery fluid.

(2) An opalescent milky-looking fluid, much more viscid than the fluid described in (1).

(3) A secretion creamy and thick, sometimes very yellow, very viscid and sometimes appearing almost gelatinous.

The larger number of antenatal secretions examined belong to classes (2) and (3), and of these by far the larger number belong to class (2). Both these solutions coagulate on heating.

Engel [1911] in a paper on the biochemistry of cows' colostrum, in a short paragraph on the antenatal secretion, points out that Houdet [1894] had found similarly in cows two types of secretion, a viscid and a more fluid form, but he had not been able to say at what stages in the pregnancy these two different types were produced.

Woodman, Hammond and Asdell, on the other hand, as shown above, associate the three degrees of viscosity with distinct periods of lactation, the very viscid (class 3) corresponding to the secretion obtained halfway through pregnancy.

All our samples fall within the last 2 months of pregnancy. It is therefore possible that the viscosities obtained by us may be very much less than would be shown by the secretion if we could obtain a specimen at about 5 months before birth. All three different types of secretion as regards viscosity, both in multiparae and primiparae, appear during the last 2 months of pregnancy, and at all times, varying from the day before to 2 months before birth. At present it does not seem possible to correlate these three types of secretion either with the stage of gestation or with a condition depending on the number of children borne. Some correlation seems, however, to be indicated between the chemical findings and the physical condition. This point will be referred to in connection with the different constituents of the secretion studied.

B. *Chemical results.*

Protein. Protein analyses were made on specimens from 48 women, 8 women giving specimens on more than one day, 35 giving one specimen only, and 5 giving specimens in labour or on the day of labour.

Table I. *Protein (g. per 100 ml.).*

P = primiparae, M = multiparae, L = lowest, H = highest.

	Before birth						
	Weeks 8, 7, 6, 5	Weeks 4–3	Day 21–15	Day 14–8	Day 7–2	Day before labour	In labour
P	L 2·69 H 8·97	L 4·54 H 17·5	L 6·06 H 8·64	L 5·90 H 11·09	L 4·12 H 13·46	L 5·49 H 6·43	L 9·864 H 17·34
M	L 5·36 H 11·99	L 5·17 H 10·32	L 5·74 H 16·0	L 3·94 H 9·14	L 3·89 H 12·26	L 4·24 H 19·4	L 10·87 H 12·31
Average values P + M (bracketed figures = no. of samples analysed).	8·40 (15)	9·28 (8)	9·18 (7)	7·54 (8)	8·16 (16)	8·385 (11)	12·598 (4)

	After birth							
Days ...	1st	2nd	3rd	4th	5th	6th	7th	8th
P	L 4·58 H 14·1	L 2·25 H 8·34	L 1·36 H 8·47	L 1·23 H 3·66	L 1·27 H 2·34	L 1·26 H 1·76	L 1·29 H 1·96	L 0·855 H 1·94
M	L 2·64 H 13·61	L 1·64 H 8·71	L 1·59 H 3·38	L 1·51 H 2·55	L 1·54 H 1·75	L 1·3 H 1·78	L 1·14 H 1·77	L 1·07 H 1·92
Average values P + M (bracketed figures = no. of samples analysed).	7·864 (11)	4·599 (17)	2·80 (27)	1·895 (25)	1·704 (20)	1·575 (20)	1·55 (13)	1·55 (23)

The figures for the percentage of protein of the antenatal secretion are compared with those of the protein of the milk obtained from 40 women during the 10 days after labour. 156 samples of the postnatal milk were analysed. The comparison is not made with later milk, as the comparison between the milk during the early and later days of lactation has already been recorded [Widdows *et al.*, 1930].

In our earlier work [Lowenfeld *et al.*, 1927], it was suggested that a significant difference was to be found in the protein values of the early days of lactation between multiparae and primiparae.

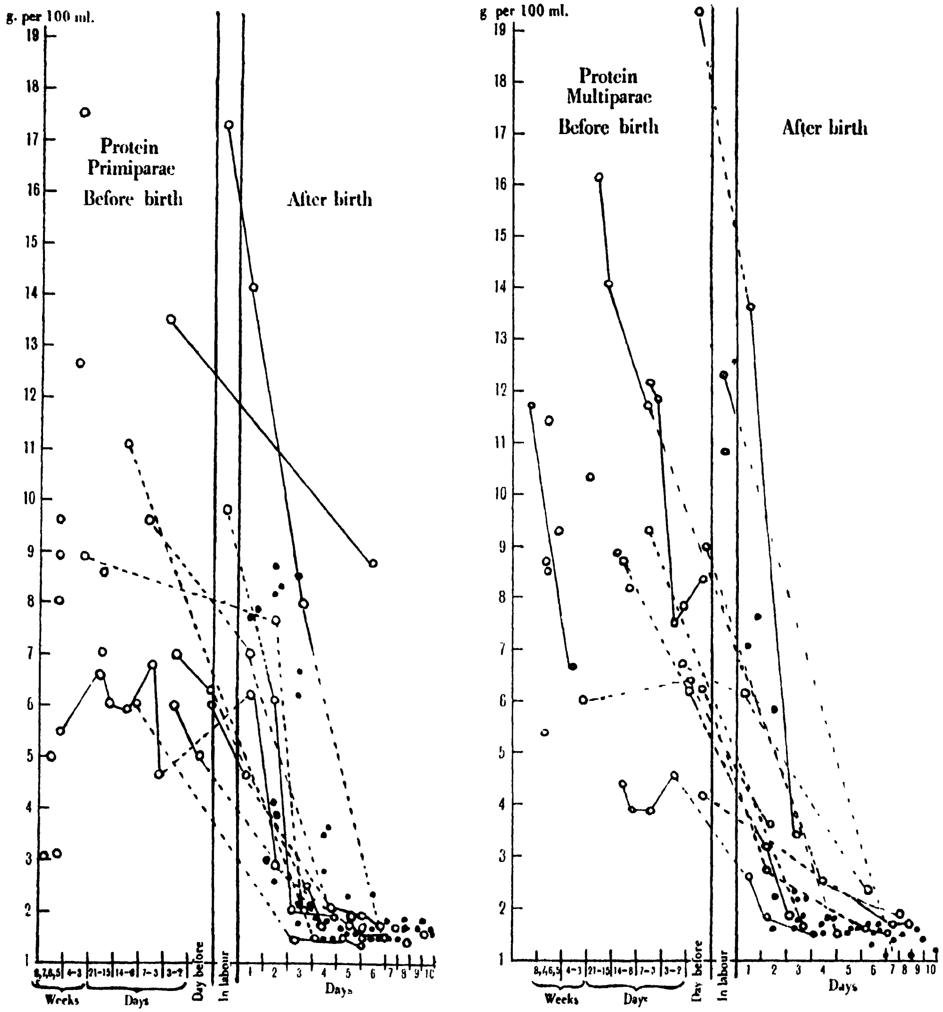

Fig. 1*a*. Fig. 1*b*.

Fig. 1. ● Represent percentages where no antenatal specimens were obtained. For clearness here percentages from same woman at different periods are not connected. o Represents percentages where both ante- and post-natal specimens were obtained. These are joined where more than one specimen from same woman was available, the connecting line being broken during periods when there were no specimens.

If the post-labour curves in the figures be considered, this suggestion that on the whole the milk of the primiparae tends to give much higher protein values than that of the multiparae on the 1st day after labour is reasonably borne out. It will be noticed that not only on the 1st day do the protein values tend to be higher, but even on the 3rd day after labour there are several values in the case of the primiparae over 6 %, whereas there are none in the case of the multiparae. On the other hand, when the values for the antenatal milk are considered, very little reliable difference can be observed between the two series of curves. This may be due (*a*) to the absence of antenatal figures in so many of the primiparae mothers who give protein values of above 5 % on the 1st to the 5th day after labour and (*b*) to the fact that the cases represented in the multiparae table show a greater number in whom it was possible to make a series of consistent observations throughout the ante- and post-natal periods.

These differences have arisen out of practical circumstances in the cases studied. In our early work the postnatal cases were obtained largely from the wards of the Obstetric Unit of the Royal Free Hospital, and as is always the case with wards of this nature, there is a heavy preponderance of primiparae in their admissions. On the other hand, the antenatal cases were studied in connection with the St Pancras Hospital, where the larger proportion of cases admitted, either as in- or out-patients of the maternity department, are multiparae, whether married or single. To decide definitely whether, as first suggested, there is any real difference between the antenatal protein values of the primiparae and the multiparae will only be possible when a large number of primiparae can be studied throughout pregnancy.

From a study of the graphs of the cases so far examined, the following facts can be ascertained.

(1) In all cases, without exception, the protein value for the antenatal secretion is higher than it is in that obtained after labour. In the secretion actually obtained in labour, in the few cases examined, the value is very high, in one case reaching the astonishing value of 17·2.

(2) The protein value for both multiparae and primiparae falls steeply during the first days after lactation, the fall, as far as our figures show being very much steeper in the case of multiparae than in that of primiparae. In the case of multiparae with a single exception, all samples were below 3 % by the 3rd day, while in the primiparae they did not reach this level till the 6th day. With only one exception, by the 3rd day all values obtained were below 2·5 % for multiparae but values above 3 % were still found on the 4th day in the case of the primiparae.

(3) One of the characteristic features obtained from a study of the curves shown equally in primiparae and multiparae, is the very great degree of variation in the protein values found in specimens of the antenatal secretion. No explanation has been found for these differences. Clemm seems to have found a similar variation in his antenatal protein results, as with the same woman on two different occasions 4 weeks before birth, he gives the percentages 2·9814 and 6·9033 respectively.

(4) The protein values appear to fall to a certain extent into two groups related to a line drawn across the curves in the neighbourhood of the value 7 %. Approximately half of the specimens examined appear to fall into a group with percentages above this level, and half below.

This tendency to fall into a group of high or low values is borne out in other constituents, but we have not as yet any suggestion to make as to the possible factors responsible for this.

Houdet [1894] in the case of cows showed that the viscid secretion gave a very high protein percentage and the more liquid secretion a low percentage. In our results practically all the very high protein percentages, those over 10 %, both in the case of the primiparae and the multiparae, are obtained from secretions class (3) which were very viscid, while the low percentages, those below 6 %, were obtained from very watery thin secretions class (1). The percentages varying from 6 to 10 % were obtained from the creamy and viscid class (2).

There are, however, exceptions to the above generalisations. For instance, in one case a secretion creamy and viscid (class 2) gave a protein percentage of 12·6 %, and another of the same consistency 12·3 %—the latter was, however, a specimen taken in labour which, as indicated by results obtained in these cases, always seems to raise the protein value. In one or two cases where the secretion was rather viscid, low protein values were obtained—*viz.* about 6 %.

A point of interest which so far we have had no opportunity of investigating, but which we hope to investigate in the future, is the type of the protein in both the antenatal and postnatal secretions.

The protein in the colostrum of cows has been investigated by, among others, Sebelien [1885], Emmerling [1888], Engel [1911], Winterstein and Strickler [1906], Southerst [1902], and Tiemann [1898] and in the antenatal secretion by Woodman and Hammond [1922; 1923], and Asdell [1925]; in human colostrum by Camerer and Söldner [1896], Birk [1910], Meigs and Marsh [1913; 1914], Pfaundler [1915], and Lowenfeld *et al.* [1927], and in the antenatal secretion by Clemm, but only so far as total protein is concerned. Czerny and Keller [1925] sum up the results on both human and cows' colostrum by stating that the "insoluble" globulin was 2–4 times, and the total "insoluble" protein 10–30 times, more concentrated than in milk.

Woodman and Hammond and Asdell show that in the case of the cow the high percentage of protein is due largely to the globulin produced at about the 5th month of pregnancy, which does not disappear from the gland until the milk is regularly removed after parturition, although the secretion of all the constituents of true milk has been taking place for some weeks.

We have noticed that all specimens of the antenatal secretion give a very deep orange colour with concentrated nitric acid, and the same colour is given by specimens on the 1st and 2nd days after parturition. From the 2nd day onwards only a very pale yellow colour is given by the milk analysed. This characteristic, combined with the fact that the secretion gives a very heavy precipitate with dilute acid, and coagulates easily on heating, may possibly be due to a high globulin content.

It is thought that it is the phenyl group in the protein which gives the deep colour with nitric acid, and from the table given by Lloyd [1926, p. 46] it will be seen that globulin contains a high percentage of the constituents containing this group. If the colour reaction with nitric acid is due to the preponderance of globulin in the antenatal secretion as well as that immediately after birth, it would seem that there is a very quick diminution of this protein with the withdrawal of the secretion after birth, as the deep orange colour disappears so rapidly.

A breast that has not had a period of lactation does not secrete as readily as one that has already done so, and it is unlikely that there will be any leakage of the secretion before parturition. From a comparison with the work on cows, it seems probable that the secretory activity of the gland in the case of the primiparae would not be as advanced as in many cases of the multiparae where stimulation to increased secretory activity would be induced by leakage. One would thus expect in the majority of cases of primiparae to find a high percentage of globulin as compared with cases of multiparae, and a smaller proportion of the constituents of normal milk both before and for a longer time after birth.

The type of protein occurring in the antenatal secretion and that immediately after birth is a point that requires investigation, but the great difficulty in attacking this problem lies in the inability to obtain sufficient of the secretion for analysis.

Sugar. The antenatal secretion was examined for sugar in 67 samples from 43 women. The number of cases whose secretion was examined on more than one day was 10, on one day only 30 and in labour 3. Between the 1st and 10th days after labour, 244 samples were analysed. In contrast to the ash and protein, as seen in the table and by the graph, the percentage of sugar is low before labour and rises very rapidly during the first days after labour. A low percentage before labour was to be expected from our earlier work [Widdows *et al.*, 1930], where it was shown that the sugar percentage was at its lowest in early milk, but had risen by the end of the 1st month to an average of a little over 7 %, from which there was little material alteration throughout the whole subsequent course of lactation. There seems to be no significant difference in the sugar values of the primiparae and multiparae. The lowest value obtained before labour was 1·1 % and the highest 4·72 %, but the usual value was between 2·5 and 3·9 %. On the 3rd day after labour, the average value in the case of both primiparae and multiparae had risen to over 6 %. In the three cases examined during labour, in the two primiparae there was a very low sugar percentage, while in that of the multipara it was high, slightly higher than the average for the six samples taken the day before labour. Whether there is really any significant difference here can only be determined by the analysis of a great many more samples, but as the difficulty of obtaining

Table II. *Sugar: average values (g. per 100 ml.).*

	Before birth						
	Weeks 9, 8, 7, 6	Weeks 4–3	Days 21–15	Days 14–8	Days 7–2	Day before labour	In labour
P	3·243	3·59	3·05	3·16	2·832	3·498	0·83
	(7)	(4)	(5)	(3)	(6)	(4)	(2)
	L 2·90	L 3·28	L 2·01	L 2·74	L 1·58	L 3·26	L 0·70
	H 3·74	H 3·89	H 3·9	H 3·76	H 3·84	H 3·84	H 0·97
M	2·79	3·94	2·44	3·94	3·13	2·76	3·165
	(6)	(3)	(4)	(5)	(10)	(6)	(1)
	L 1·78	L 3·65	L 1·76	L 2·76	L 1·10	L 2·06	—
	H 3·54	H 4·22	H 3·58	H 4·72	H 4·12	H 3·14	—
P+M	3·033	3·741	2·78	3·645	3·02	3·054	1·110
	(13)	(7)	(9)	(8)	(16)	(10)	(3)
	L 1·78	L 3·28	L 1·76	L 2·74	L 1·1	L 2·06	L 0·70
	H 3·74	H 4·22	H 3·9	H 4·72	H 4·12	H 3·84	H 3·165

	After birth							
Days ...	1st	2nd	3rd	4th	5th	6th	7th	8th, 9th and 10th
P	2·86	4·68	6·22	6·67	6·95	7·175	7·02	7·27
	(8)	(17)	(16)	(19)	(15)	(15)	(11)	(19)
	L 1·08	L 2·61	L 3·55	L 6·67	L 5·38	L 6·11	L 6·53	L 5·67
	H 4·7	H 6·4	H 7·77	H 7·69	H 7·58	H 8·36	H 7·14	H 8·27
M	3·58	4·37	6·396	6·68	6·92	7·02	7·35	7·33
	(6)	(20)	(21)	(19)	(13)	(13)	(11)	(20)
	L 2·46	L 1·49	L 4·65	L 4·98	L 4·77	L 5·97	L 6·66	L 6·00
	H 6·26	H 6·9	H 7·4	H 7·64	H 7·87	H 7·78	H 7·76	H 8·10
P+M	3·17	4·51	6·32	6·67	6·94	7·10	7·18	7·30
	(14)	(37)	(37)	(38)	(28)	(28)	(22)	(39)
	L 1·08	L 1·49	L 3·55	L 4·98	L 4·77	L 5·97	L 6·53	L 5·67
	H 6·26	H 6·9	H 7·77	H 7·69	H 7·87	H 8·36	H 7·76	H 8·27

such samples is evident, the authors felt it was of interest to record these results.

There is, however, this point to notice, that in the case of the multipara G. the sample analysed was taken about 7 hours before the birth of the child, while in the case of both of the primiparae, the sample was taken at an earlier stage in labour. One, W., was taken at the beginning of labour where the labour lasted 2 days, and the other J. was taken between 16–17 hours before the birth of the child. Whether in the case of the multipara the value of the sugar percentage was lower at the beginning of labour, and at the time of taking was beginning to rise as it always does directly after labour, or whether there is really a difference between the primiparae and multiparae can only be decided by further work. In no case yet have we been able to make a consecutive series of analyses beginning a few days before and continued through labour to a few days after labour. Only from such analyses could any definite conclusions be drawn.

On considering the highest and lowest values in the average of percentage for sugar for the different periods, in Table II it will be seen that the primiparae and multiparae seem to divide themselves into two groups as in the case of protein and ash, (1) those starting with a percentage of sugar before labour of 2 % and rising during the second week after labour to 6 %; (2) those starting before labour with a percentage of about 3·8 % and rising in the same period after labour to 8 %. The quicker rise to a higher figure after labour is more manifest in the groups starting with higher sugar before labour.

On examining the different classes of fluid, it was found that the percentage of sugar for the very viscid class 3 (20), the opalescent and viscid milky fluid class 2 (43), the watery secretion class 1 (14) gave respectively average values of 2·717, 3·45 and 3·585 g. Although the highest individual value 5·02 % was found among the less viscid type class 2, yet in the case of the watery secretion the values, except in one case, were all well over 3 % and in some cases over 4 %, the lowest being 2·79 %. In the other types of secretion there were many values between 2 and 3 %.

Ash. The antenatal secretion of 50 women has been examined for ash. A sample on more than one day was obtained from 9 women and on one day only from 36 women. Five samples were obtained during labour.

There is no significant difference to be observed between the multiparae and the primiparae.

The ash percentage is consistently high throughout the antenatal period. Out of 74 samples analysed, only in two instances did it fall below 0·3 %. The majority of cases give a percentage between 0·5 and 0·68, except in two samples from one particular patient on two consecutive days when the percentage obtained was 0·8. In the five cases in labour the percentage is over 0·6.

After labour there is a steady decline in the percentage of the ash from over 0·5 % on the 1st day to between 0·2 and 0·3 % after the 1st week.

The specimens from both the primiparae and the multiparae seem to fall into two groups:

(1) Those starting in the antenatal period at 0·5 % and declining gradually to 0·16 % 10 days after labour.

(2) Those starting about 0·68 % and gradually declining in the same period of time to about 0·34 %.

The graphs for ash and protein run almost parallel. The graphs show rather irregular figures before labour, falling gradually but persistently after labour to a more or less constant level.

Table III. *Ash: Average values in g. per 100 ml.*

	Before birth						
	Weeks 8, 7, 6, 5	Weeks 4–3	Days 21–15	Days 15–8	Days 7–2	Day before labour	In labour
P	0·54	0·53	0·511	0·675	0·556	0·475	0·70
	(8)	(4)	(6)	(4)	(5)	(4)	(3)
	L 0·5	L 0·44	L 0·31	L 0·52	L 0·40	L 0·39	L 0·68
	H 0·6	H 0·60	H 0·64	H 0·80	H 0·66	H 0·62	H 0·74
M	0·57	0·38	0·56	0·49	0·535	0·565	0·62
	(8)	(3)	(3)	(6)	(9)	(7)	(2)
	L 0·48	L 0·28	L 0·52	L 0·28	L 0·30	L 0·40	L 0·62
	H 0·68	H 0·52	H 0·60	H 0·56	H 0·60	H 0·60	H 0·62
P+M	0·55	0·46	0·53	0·566	0·543	0·532	0·668
	(17)	(7)	(9)	(10)	(14)	(11)	(5)
	L 0·48	L 0·28	L 0·31	L 0·28	L 0·30	L 0·39	L 0·62
	H 0·68	H 0·60	H 0·64	H 0·80	H 0·66	H 0·62	H 0·74

	After birth							
Days ...	1st	2nd	3rd	4th	5th	6th	7th	8th, 9th and 10th
P	0·59	0·365	0·34	0·325	0·29	0·28	0·26	0·257
	(5)	(7)	(12)	(18)	(13)	(14)	(12)	(24)
	L 0·50	L 0·26	L 0·24	L 0·22	L 0·23	L 0·17	L 0·14	L 0·16
	H 0·72	H 0·60	H 0·66	H 0·44	H 0·36	H 0·42	H 0·38	H 0·34
M	0·53	0·39	0·32	0·31	0·29	0·27	0·265	0·259
	(6)	(9)	(14)	(11)	(10)	(12)	(11)	(22)
	L 0·40	L 0·29	L 0·20	L 0·20	L 0·20	L 0·20	L 0·16	L 0·16
	H 0·62	H 0·64	H 0·46	H 0·54	H 0·38	H 0·42	H 0·38	H 0·33
P+M	0·556	0·382	0·33	0·32	0·29	0·275	0·264	0·258
	(11)	(16)	(26)	(29)	(23)	(26)	(23)	(46)
	L 0·40	L 0·26	L 0·20	L 0·20	L 0·20	L 0·17	L 0·14	L 0·16
	H 0·72	H 0·64	H 0·66	H 0·54	H 0·38	H 0·42	H 0·38	H 0·34

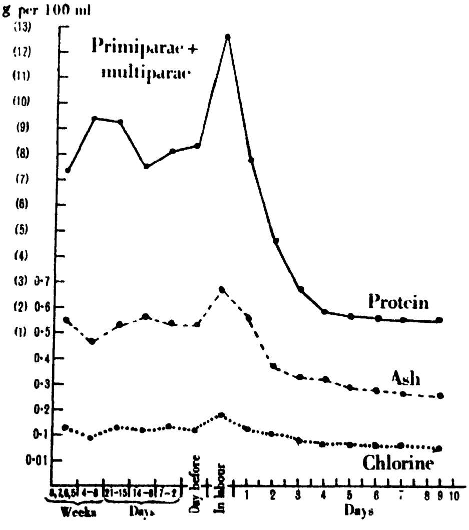

Fig. 2.

As regards the relation of the ash content to the physical properties of the secretion, in the case of cows, Houdet, according to Engel [1911], found that in the viscid secretion there were only traces of salts and in the more fluid about 0·36 %.

In the case of the human secretion, a study of our figures does not seem to show any striking connection between the three classes of fluid and the ash values found. In the very viscid fluids (20) class 3, there was only one example giving a low ash 0·3 %, in the less viscid secretion (46) class 2 and the watery secretion (15) class 1 both high and low values occurred, the high values largely preponderating in both. The averages for the three types respectively were 0·586, 0·518 and 0·49 %. The high or low values do not seem to occur at any definite time during the antenatal period, both high and low values are found at all times from 8 weeks before and up to the day of birth, although at all periods the high values largely predominate.

Calcium. Calcium has been investigated in the antenatal secretion of 49 women, in 36 cases on one day only, 9 on more than one day and 4 in labour, 74 samples in all having been analysed. The results so obtained are compared with those obtained from the secretion during the 10 days immediately after labour, of which 215 examples were examined.

On the whole the percentage of calcium in the antenatal secretion is low, although occasionally a high value is obtained. In the few specimens obtained during labour, the average shows a decrease in the percentage at this time, rising immediately after labour and continuing to rise gradually during the next 10 days.

It has been shown already [Widdows *et al.*, 1930] that the calcium value rises gradually to a maximum during the first 4 months after birth, and then falls very slowly during the 5th to the 7th months, and more rapidly from the 7th to the 10th months.

We have in our earlier work on two occasions [Lowenfeld *et al.* 1927; Widdows *et al.*, 1930] in agreement with other workers [Hunaeus, 1909; Bahrt and Edelstein, 1910] emphasised the fact that the differences between individuals in the calcium content of the milk are much greater than the variations from day to day in any one individual.

This makes the interpretation of results difficult where only a few samples of the secretion can be obtained, and where it is not possible in the same women to obtain samples for the few days consecutively before and after the period in question, as for example, in the cases of samples obtained from women in labour.

From Table IV it will be seen that in the primiparae the average percentage of calcium oxide the day before labour is 0·0366, during labour 0·0305, and on the 2nd day after labour 0·0376. Only three samples were obtained during labour, two from the same woman gave low calcium oxide values of 0·0254 and 0·0264 %, whilst the one from the other gave higher values of 0·0396 %. Four samples were obtained on the day before labour but from different women from those in labour. One gave a high value for the calcium oxide 0·0548 %, higher than the highest value during labour, 0·0396 %, and the others the low values of 0·0272, 0·033 and 0·0311 %. The lowest value before labour, 0·0272 %, is also higher than either of the low values 0·0254 and 0·0264 % during labour.

In the case of the multiparae, where the secretion was examined in the case of only two women during labour, the percentage of calcium oxide in one sample was 0·0258 g., and in the other 0·0234 g. both low values, but in the seven samples the day before birth the highest percentage of calcium oxide in one sample was 0·041 g. and the lowest 0·0224 g. Here also the highest value before labour, 0·041 g., is higher than the highest value during labour, 0·0258 g., on the other hand, the lowest value before labour, 0·0224 g., is lower than that of the lowest value, 0·0234 g., during labour. Again, these values were not those of samples taken from the same women as those whose samples were taken in labour, and the value 0·0224 g. was the only one out of seven lower than the value in labour, 0·0234 g.

In these circumstances, the average values are probably a better criterion of the relative changes occurring throughout the ante- and post-natal periods studied.

From our work on milk in the early and later days of lactation it was realised that some women throughout lactation had consistently a higher calcium value for their milk than others, and it was therefore not surprising to find that this was also the case in the antenatal secretion. For instance, in one case M. where in the antenatal period 10 days before birth four samples were taken, the calcium oxide percentage varied between 0·027 g. and 0·025 g., in the postnatal period the value gradually rose to 0·0388 g., whereas in another case R., where

Table IV (*a*).

Calcium oxide.

Average values—g. per 100 ml.

A. Before birth.

	Weeks 8, 7, 6, 5	Weeks 4–3	Days 21–15	Days 14–8	Days 7–2	Day before labour	In labour
P	0·030	0·0347	0·024	0·0285	0·0326	0·0366	0·0305
	(8)	(5)	(6)	(4)	(5)	(4)	(3)
	L 0·013	L 0·26	L 0·0198	L 0·026	L 0·022	L 0·0272	L 0·0254
	H 0·043	H 0·042	H 0·031	H 0·033	H 0·055	H 0·0548	H 0·0396
M	0·0294	0·035	0·0398	0·030	0·031	0·030	0·0246
	(8)	(3)	(3)	(6)	(9)	(7)	(2)
	L 0·0197	L 0·031	L 0·025	L 0·025	L 0·0235	L 0·0224	L 0·0234
	H 0·034	H 0·0395	H 0·045	H 0·044	H 0·042	H 0·041	H 0·0258
P + M	0·030	0·035	0·0293	0·0298	0·0305	0·0325	0·0281
	(17)	(8)	(9)	(10)	(14)	(11)	(5)
	L 0·013	L 0·026	L 0·0198	L 0·025	L 0·022	L 0·022	L 0·0234
	H 0·043	H 0·042	H 0·045	H 0·044	H 0·055	H 0·054	H 0·0396

Phosphorus pentoxide.

Average values—g. per 100 ml.

P	0·0074	0·0143	0·0169	0·0128	0·0187	—
	(6)	(1)	(2)	(2)	(4)	—
	L 0·005	—	L 0·011	L 0·0119	L 0·0146	—
	H 0·0119	—	H 0·0229	H 0·0137	H 0·0204	—
M	0·0189	0·020	—	0·0138	0·0119	0·01422
	(3)	(2)	—	(4)	(5)	(5)
	L 0·0133	L 0·0172	—	L 0·0111	L 0·0078	L 0·0114
	H 0·0292	H 0·023	—	H 0·0143	H 0·014	H 0·0153
P + M	0·0118	0·0181	0·0169	0·0132	0·0149	0·0142
	(10)	(3)	(2)	(6)	(9)	(5)
	L 0·005	L 0·0143	L 0·011	L 0·0111	L 0·0078	L 0·0114
	H 0·0292	H 0·023	H 0·0229	H 0·0143	H 0·0204	H 0·0153

$\frac{\text{Calcium oxide}}{\text{Phosphorus pentoxide}}$ ratio.

3·21	2·1	1·54	2·41	2·2	2·08
(10)	(3)	(2)	(8)	(8)	(5)

Average ratio for 4 weeks before birth = 2·17.

Table IV (*b*).

Calcium oxide.

Average values—g. per 100 ml.

B. After birth.

Days...	1st	2nd	3rd	4th	5th	6th	7th	8th, 9th, and 10th
P	0·0284	0·0376	0·039	0·041	0·0436	0·042	0·041	0·041
	(5)	(10)	(15)	(18)	(11)	(14)	(11)	(34)
	L 0·025	L 0·020	L 0·025	L 0·030	L 0·033	L 0·032	L 0·032	L 0·025
	H 0·034	H 0·062	H 0·049	H 0·069	H 0·054	H 0·049	H 0·059	H 0·069
M	0·035	0·0359	0·041	0·0416	0·042	0·044	0·0397	0·0395
	(7)	(9)	(15)	(14)	(11)	(11)	(10)	(19)
	L 0·023	L 0·024	L 0·021	L 0·023	L 0·029	L 0·034	L 0·034	L 0·0245
	H 0·046	H 0·054	H 0·059	H 0·048	H 0·064	H 0·059	H 0·045	H 0·050
P+M	0·0323	0·0368	0·0398	0·0414	0·042	0·0425	0·041	0·041
	(12)	(19)	(30)	(32)	(22)	(26)	(21)	(53)
	L 0·023	L 0·020	L 0·021	L 0·023	L 0·029	L 0·032	L 0·032	L 0·0245
	H 0·046	H 0·062	H 0·059	H 0·069	H 0·064	H 0·059	H 0·059	H 0·069

Phosphorus pentoxide.

Average values—g. per 100 ml.

P	0·0215	0·0212	0·0252	0·0276	0·0377	0·0376	0·0365	0·0377
	(2)	(6)	(6)	(8)	(7)	(7)	(7)	(27)
	L 0·0204	L 0·0145	L 0·0145	L 0·0189	L 0·0229	L 0·027	L 0·0332	L 0·0251
	H 0·0227	H 0·0263	H 0·0355	H 0·0355	H 0·0475	H 0·043	H 0·0437	H 0·056
M	0·0265	0·0265	0·030	0·031	0·0339	0·0392	0·0371	0·0408
	(2)	(5)	(9)	(11)	(9)	(7)	(8)	(12)
	L 0·0145	L 0·0183	L 0·0129	L 0·0138	L 0·0189	L 0·0297	L 0·0229	L 0·0306
	H 0·0387	H 0·0356	H 0·0424	H 0·045	H 0·0475	H 0·0504	H 0·0504	H 0·0504
P+M	0·024	0·0236	0·0281	0·0296	0·0356	0·0384	0·0368	0·0384
	(4)	(11)	(15)	(19)	(16)	(14)	(14)	(39)
	L 0·0145	L 0·0145	L 0·0129	L 0·0189	L 0·0189	L 0·027	L 0·0229	L 0·0251
	H 0·0387	H 0·035	H 0·0424	H 0·045	H 0·0475	H 0·504	H 0·0504	H 0·056

$\frac{\text{Calcium oxide}}{\text{Phosphorus pentoxide}}$ ratio.

1·4	1·32	1·59	1·45	1·23	1·01	0·99	1·36
(3)	(3)	(13)	(14)	(9)	(10)	(12)	(21)

Average ratio for first 7 days = 1·27.

three samples were obtained, one a month, one 3 weeks and one 4 days before birth, the values were over 0·04 %, and they remained high after birth, rising on the 4th day after labour to 0·048 %.

From these average values, it is concluded (*a*) that the calcium percentage in the secretion before birth rarely rises to much over 0·03, and in many cases is much less, and (*b*) that it is at its lowest value during labour and steadily rises after labour towards its maximum.

There does not appear to be any difference in the calcium values in the cases of the primiparae and the multiparae.

Phosphorus. The antenatal mammary secretions of 27 women were examined for phosphorus, and 34 samples were analysed, of which 23 represented single specimens from 23 different women and 11 were samples from 4 women, one providing 4, one 3, and two 2 samples on different days. 132 samples of milk from the 1st to the 10th day after birth were analysed, and the results compared with those obtained from the antenatal secretion.

The percentage of phosphorus in the secretion is very low throughout the antenatal period studied, but begins to rise immediately after labour. It has been previously shown that the maximum value for the phosphorus pentoxide percentage is not realised till the end of the 4th month after parturition. Before birth, the highest percentage of phosphorus pentoxide, except in one case of a multipara giving 0·029, is only just over 0·02, and in many cases the percentage is much lower.

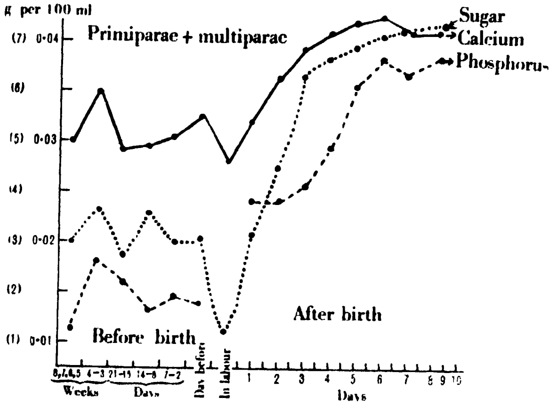

Fig. 3.

Ca/P ratio. On examining the graphs for calcium oxide and phosphorus pentoxide at the different stages both before and after labour, it will be seen that they follow each other very closely, the phosphorus values being at a much lower level before birth than those of the calcium. In the case of the calcium oxide the average percentage before parturition is over 0·03 or approximating to it, the highest value for each period being over 0·04, while, as stated above, in only one case does the value rise to 0·029 in the case of the phosphorus pentoxide. After birth too the average value for the calcium oxide percentage has risen to over 0·04 on the 4th day, while in the case of the phosphorus pentoxide, on the 10th day the value is still lower than that of the calcium.

Schlossmann [1904] thought that a high protein value was associated with a high phosphorus value, but our work does not seem to support this view. Our protein values are highest before and just after birth, while our phosphorus values are low during all these periods.

In our work on later milk it was shown that the percentage of calcium oxide in milk throughout lactation was usually greater than that of phosphorus pentoxide, and that the average ratio of these percentages did not show a great variation. The average ratios during the periods 1–2 weeks, 1 month, 2–4 months and 5–10 months were 1·35, 1·34, 1·42, 1·25 respectively. The individual variations however were shown to be greater, varying from 0·81 to 2. McCollum *et al.* [1921] had pointed out the importance of this ratio, and in our paper [Widdows *et al.*, 1930] this ratio was discussed and an explanation suggested.

In the case of the antenatal secretion this ratio was also examined and as the percentage of phosphorus pentoxide before birth relative to that of calcium oxide was considerably lower than it is after birth, this ratio necessarily has a higher value. The average value for the ratio for the whole period from 8 weeks to the day before birth is 2·46 and for the different periods considered the average ratio varied from 1·5 to 3·21. The average value for the first 7 days after birth is 1·27.

One would expect the ratio of calcium to phosphorus in the antenatal secretion to be different from, and more variable than, that in the milk after birth, partly on account of the fact that the secretion has no practical value as a food for the infant at that time, and also because the proportions of the calcium and the phosphorus present must depend on several factors. These factors are:

(1) The demand of the foetus for these elements at the stage of gestation under consideration.

(2) The necessity of a supply of these elements (which are constituents of normal milk) to the secretion gradually developing to form the fluid which is the perfect food for the newborn infant.

(3) The capacity of the blood supply of the mother to provide all the calcium and phosphorus required both for the foetus and the secretion without drawing unduly on her own reserves.

Since in our work we have only been able to study the composition of the secretion during the last 2 months before parturition, it will here only be possible to consider that period.

Bar [1907] had made an exhaustive study of the nutrition of the foetus during normal pregnancy. In this work he has shown that the need of the foetus for both phosphorus and calcium is usually very small during the first part of gestation but increases enormously during the last 2 months. During this latter period the foetus demands from the maternal organism 0·58 g. of phosphorus in the form of the pentoxide and 0·638 g. of calcium in the form of the oxide per day, while from the 4th to the 6th month only 0·046 g. of phosphorus pentoxide and 0·083 g. of calcium oxide are required per day. There is, therefore, a heavy drain on the mother by the foetus at this period for these two elements, so that the secretion developing to form the normal milk will only, one may suppose, obtain its necessary supply after the needs of the foetus are satisfied.

Both in the case of phosphorus and calcium there is a large retention towards the end of the pregnancy as shown by the decreased urinary output of these elements, although in the case of phosphorus Bar states that the proportion of organic phosphorus in the urine may be very slightly increased.

Since our ratio of calcium oxide to phosphorus pentoxide in the mammary secretion is higher before than it is after parturition, it is probable that there is more calcium relatively to phosphorus available for the secretion from the parent organism before than there is after birth.

Bar points out, from his work on dogs, that during gestation the mother can remove from the food the phosphorus elements necessary for the development of the foetus, and, provided the ration is sufficient, does not draw on her own capital of phosphorus.

He has also shown that phosphorus assimilation follows closely that of nitrogen. At the beginning of gestation there is a retention of both above the needs of the foetus, followed by a period of excretion probably due to the saturation of the organism by these elements. During the last weeks there is a very active retention to meet the heavy demands of the foetus.

In the case of nitrogen, throughout the whole of pregnancy, its assimilation seems to be good so that Bar remarks that in women in good health free to regulate their own diet it is common to observe a retention of nitrogen above that required for the foetus, its appendages, and the development of the uterus and the breasts, and that the nitrogen retained goes to increase the nitrogen capital of the mother. Consequently pregnancy appears to be a period of gain of nitrogen to the maternal organism. This would account for the high nitrogen content of the mammary secretion both before and after parturition which has been emphasised in the preceding pages.

Bar showed also that although the assimilation of phosphorus follows the same course as that of nitrogen, its retention is by no means as good, and that there may be a gain of nitrogen where at the same time there is a loss of phosphorus.

Towards the end of its intra-uterine life, the demand of the foetus for phosphorus is greater than it is for nitrogen, but, in spite of this, in the ration absorbed the proportion of phosphorus may be small—further in the case of women Bar has observed that the utilisation of the absorbed phosphorus ration has been frankly mediocre. This he points out may be due among other things to the phosphorus being present largely in the inorganic form owing to an excess of mineral bases, and thus passing into the state where it is of no value for building up the tissues of the foetus.

It is evident from Bar's work that the parent organism will often have as much as it can do to supply the foetus at the end of gestation with its necessary phosphorus, and that therefore there can only be a small amount available for the mammary secretion that is forming. The fact that the phosphorus percentage in this secretion is low both before and immediately after parturition has perhaps here an explanation, especially as the women whose secretions were examined all came from the poorer classes where the food ration is likely to be poor in phosphorus.

In the case of the calcium Bar has shown that four-fifths of the calcium which the foetus contains at full term is acquired during the last 2 months of gestation. During the early part of pregnancy he has also shown that there is mobilisation of calcium, though this is not required by the foetus at that time. This is eliminated largely by the faeces and the urine, but at the end of gestation, when the needs of the foetus become intense, this elimination suddenly diminishes.

It appears from Bar's work that there is always calcium available during pregnancy, more than sufficient for the needs of the foetus, some always being derived from the maternal organism. It is therefore not surprising that the antenatal mammary secretion, as we have found, contains a larger percentage of calcium relative to that of the phosphorus than is found in the milk secreted normally after birth.

From a study of the calcium in blood of pregnant women, it has been shown by de Wesselow [1922] and Widdows [1923] that, in spite of the heavy demands made by the foetus during the last 2 months before birth, the blood tends to maintain a fairly constant value for calcium oxide, tending to decrease during the 8th and 9th months and to rise again immediately after parturition. Among other workers, Bar [1907] and Lamers [1912] found the blood calcium higher in pregnant than in non-pregnant women, Kehrer [1920] found it definitely low in pregnant women, and Jansen [1918, 1, 2] found it little altered. The above discrepancies may be in part accounted for by the fact that de Wesselow and Widdows analysed the serum and the other workers the whole blood.

In the case of the whole blood the calcium is not distributed evenly between the plasma and the corpuscles and so, unless the proportion of the corpuscles to plasma is ascertained, the true value for the calcium is not obtained. De Wesselow [1922] found also that in the case of phosphorus there was a tendency for its value to fall during the last 2 months of gestation, rising after parturition and during lactation to a figure definitely above that of the non-lactating female.

These facts bear out and we think explain the reasons for the calcium value of the antenatal mammary secretion being high relatively to the phosphorus before parturition.

Chlorine. No record has been given in any previous papers of our results on the percentage of chlorine either in early or late milk, although estimations have been made during these periods.

The method of estimation for the chlorine was that of Patterson [1928]. Our results on the whole agree with those of Sissons and Denis [1921] who have made a fairly comprehensive study of this element in postnatal milk. Our average for 80 samples taken from different women at all stages of lactation from 2 weeks to 42 weeks after birth is 32·1 mg. per 100 ml. of milk. The average given by Sissons and Denis is 38·1. These investigators found that in normal women after the 1st week of lactation there was a relatively narrow range of variation from 30 to 50 mg. of chlorine per 100 ml. of milk. We have found that the range in our cases was from 20 to 50 mg., that 25 % of our cases varied from 30 to 50 mg. and 48·75 % from 20 to 30 mg. Out of the 80 samples analysed we had only 10 over 50 mg. per ml. Sissons and Denis found that specimens of milk in which the chlorine concentration was over 50 mg. per ml. were from mothers who were giving an inadequate amount of milk for the growth of the infant. Our 10 cases where the percentage of chlorine was over 50 mg. per 100 ml. and not exceeding 58 mg. do not seem to support this contention, as in all cases, except one, the supply of milk was plentiful and the child was doing well. In

Table V. *Chlorine.*

Average values—g. per 100 ml.

	Before birth						
	Weeks 8, 7, 6, 5	Weeks 4–3	Days 21–15	Days 14–8	Days 7–2	Day before labour	In labour
P	0·1082	0·0868	0·1313	0·1240	0·1263	0·400	0·20
	(8)	(4)	(5)	(2)	(6)	(3)	(1)
	L 0·072	L 0·0236	L 0·0624	L 0·075	L 0·0588	L 0·072	—
	H 0·152	H 0·127	H 0·163	H 0·173	H 0·186	H 0·153	—
M	0·1414	0·0866	0·013	0·102	0·1349	0·1182	0·1644
	(7)	(3)	(2)	(4)	(10)	(7)	(2)
	L 0·0454	L 0·072	L 0·045	L 0·0776	L 0·0685	L 0·0936	L 0·133
	H 0·276	H 0·103	H 0·181	H 0·161	H 0·210	H 0·1583	H 0·1958
P+M	0·123	0·086	0·126	0·111	0·132	0·1128	0·1762
	(16)	(8)	(7)	(6)	(16)	(10)	(3)
	L 0·0454	L 0·0236	L 0·045	L 0·075	L 0·0588	L 0·072	L 0·20
	H 0·276	H 0·127	H 0·180	H 0·173	H 0·210	H 0·158	H 0·133

	After birth							
Days...	1st	2nd	3rd	4th	5th	6th	7th	8th, 9th and 10th
P	0·11	0·1078	0·0743	0·0637	0·0543	0·0541	0·0558	0·0488
	(12)	(16)	(14)	(22)	(16)	(17)	(11)	(32)
	L 0·058	L 0·0588	L 0·033	L 0·033	L 0·020	L 0·0169	L 0·023	L 0·0248
	H 0·2335	H 0·1875	H 0·1158	H 0·137	H 0·073	H 0·116	H 0·096	H 0·094
M	0·1289	0·0975	0·0755	0·0563	0·0498	0·0509	0·0510	0·0415
	(9)	(24)	(30)	(22)	(21)	(18)	(11)	(35)
	L 0·060	L 0·0582	L 0·036	L 0·026	L 0·026	L 0·0248	L 0·0327	L 0·0291
	H 0·232	H 0·185	H 0·1440	H 0·0927	H 0·0927	H 0·077	H 0·0779	H 0·0685
P+M	0·1187	0·1016	0·0755	0·05997	0·0518	0·0525	0·0534	0·0436
	(21)	(40)	(44)	(44)	(37)	(35)	(22)	(69)
	L 0·058	L 0·0582	L 0·033	L 0·026	L 0·020	L 0·0169	L 0·023	L 0·0248
	H 0·233	H 0·1874	H 0·144	H 0·137	H 0·092	H 0·116	H 0·096	H 0·094

this one case, as well as in one additional case where the content of chlorine was 72 mg. per 100 ml., it is true the supply of milk was scanty, but as, in both cases, artificial feeding had been adopted and the child was doing well, it is not possible to say whether these cases support the findings of Sissons and Denis.

It may be that where the milk is insufficient for the nourishment of the child the chlorine percentage may be high, but it does not seem that a content of chlorine over 50 mg. per 100 ml. is necessarily accompanied by a scanty supply of milk and an ill-nourished child.

We found, as did Sissons and Denis, extreme variations in the chlorine contents of specimens of milk collected from women in the same period of lactation. In the 4th week of lactation there was a variation from 23·9 mg. per 100 ml. to 52·2 mg. per 100 ml. and specimens with high and low contents were found to occur at all stages of lactation. In one case, a 4-week specimen gave 23·9 mg. per 100 ml. chlorine, and in another 52·2 mg. per 100 ml., while two 19-week specimens gave respectively 29·7 mg. and 42·2 mg. per 100 ml. It was noticed that only during the last weeks of lactation, with one exception, did the value even fall below 20 mg. per 100 ml.

There is a tendency for a high concentration of chlorine during the 1st week after birth. Sissons and Denis found that it varied from 158 mg. to 42 mg. per 100 ml. Our average variation, examined between the 1st and 7th days after parturition, was from 118 mg. to 51 mg. per 100 ml. for 243 specimens, and the average value for the 8th, 9th and 10th days from 69 samples was 43·6 mg. per 100 ml. Our percentages during this period varied from 233 mg. to 20 mg., one sample giving a value of 16·9 mg.

Sixty-three samples of antenatal secretion have been investigated for their chlorine contents. The percentage of chlorine is strikingly higher throughout the whole antenatal period examined, and seems to run parallel with that of the protein and ash.

The average given by the 63 samples is 118 mg. per 100 ml., the same as the average value found for the 1st day after labour. The range of values is from 276 mg. to 45 mg. per 100 ml. In the whole 63 samples there are only three, including one of a primipara in the 3rd to 4th week before birth giving 23·6 mg., which give a value for chlorine less than 50 mg. per 100 ml., and 57 % of the samples analysed gave over 100 mg. per 100 ml. Since the value for the percentage of chlorine is high immediately after birth, it was to be expected from analogy with the protein and ash values that the chlorine value before birth would also be high, and, as shown, this expectation was realised since both the average and individual values in the antenatal secretion are considerably above those found in the early postnatal milk.

Three specimens from women in labour were investigated and show a much higher chlorine value than the average for the day before or the day after birth. In the case of the primipara the value 200 mg. per 100 ml. in labour is above the values for all the three samples taken the day previous to birth, and in the case of the two multiparae the higher value is above the values for all the seven samples taken the day before birth, while the lower one is higher than five, equal to one and lower than one. The women giving the samples in labour are none of them women from whom samples had been obtained the day before birth.

The same extreme variations were noticed in the chlorine content of specimens of the antenatal secretion collected from different women at the same period of lactation as had already been noted in the cases during later lactation. There seems no significant difference between the results obtained from primiparae and multiparae.

The values for the chlorine in the antenatal period, as already noticed, are always high, but the highest values do not necessarily correspond to the highest ash values.

The curve for chlorine follows that of the protein and the ash, but a study of the figures for chlorine does not show any striking relationship between the viscosity of the secretion and the chlorine value. Both high and low values occur in all three classes of the secretion. Where, however, several samples of the secretion from the same woman were taken at different stages during the antenatal period, it was noticed that, if the ash value varied, the increase or decrease coincided with a corresponding variation in the chlorine value. In one case, for instance, where the value of the ash rose from 0·44 to 0·52 %, the chlorine value rose from 45 mg. to 48 mg. per 100 ml.

The average values for the very viscid (21) class 3, the less viscid (40) class 2, and the watery (14) class 1 secretions are respectively 0·106, 0·1158 and 0·1307 g. per 100 ml., the watery secretion showing the highest average. It has not been possible so far to estimate the alkali metals and magnesium, and it may be that the chlorine percentages are related to these elements.

SUMMARY.

1. A total of 78 specimens from 53 women of the antenatal secretion for periods from 2 months before parturition up to the time of labour and in labour were analysed. The constituents studied were protein, sugar, ash, calcium, phosphorus and chlorine.

2. The results have been compared with work on postnatal milk during the first 10 days after labour and also with similar work on cows.

3. In all cases results suggested from our previous work as to the probable composition of the antenatal secretion have been obtained, suggestions have been made to account for certain experimental facts, but there are points for which at present no explanation can be offered.

4. A comparison of the relative proportions of the main ingredients of the fluid secreted by the mammary gland before and after parturition shows that a true difference in secretory activity is involved and not merely a difference in concentration.

5. A study of the physical properties of the antenatal secretions shows them to fall into three classes: (1) a beige watery fluid, (2) an opalescent more viscid fluid and (3) a very viscid cream to bright yellow fluid. All three forms were found during the last 2 months of pregnancy and could not be correlated either with absence or presence of previous lactation or, as in the case of cows, with the period of pregnancy.

6. In all cases the protein value for the antenatal secretion is higher than for that obtained after labour, and these values tend to fall into two groups with a mean value of 7 %.

The protein values of the secretion for the first few days of lactation are higher in the primiparae than in the multiparae. There appears to be a definite relationship between the viscosity of the secretion and the percentage of contained protein.

7. In the case of the sugar low values are obtained before labour, the mean value lying between 2·5 and 3·9 %.

In sugar content, as with protein, the samples tend to fall into two groups, 2 % rising to 6 % and 3·8 % rising to 8 %.

A definite relation exists between the viscosity of the secretion and the sugar percentage.

8. The percentage of ash in the antenatal secretion is always high, and in the five cases examined in labour the percentage was over 6 %. As in the case of protein irregular figures are shown before labour, falling steadily and persistently after labour to a more or less constant level.

9. In the case of calcium, while the fact that very considerable differences exist between the averages for individual women obscures the interpretation of the figures, yet from the average values the conclusion may be drawn that the percentage of calcium oxide is lowest during labour, rarely rising above 0·03 before labour, and steadily rising to a maximum after labour.

10. The phosphorus percentages in the antenatal period are very low, the highest percentage (P_2O_5) being only just over 0·028. The curves for phosphorus follow very closely those for calcium.

The calcium/phosphorus ratio before parturition is higher than that obtained after parturition. This is consistent with the work of Bar on the needs of the developing foetus for this element.

11. The percentage of chlorine is consistently very strikingly higher in the antenatal than in the postnatal period. There appears to be no clear relationship between the viscosity of the fluid and the percentage of chlorine present.

The curves run parallel for chlorine, protein, and ash.

In conclusion we have to express our thanks to Dr Harding and Sister Griffin at the St Pancras Hospital and Dr Macgregor at the Shoreditch Carnegie Welfare Settlement for facilities for obtaining the material for the above investigation. We wish to thank Prof. Winifred Cullis for valuable criticism and advice, and the Medical Research Council and the Waller Research Fund for grants towards the expenses of the work.

REFERENCES.

Asdell (1925). *J. Agric. Sci.* **15**, 358.
Bahrt and Edelstein (1910). *Jahrb. Kinderheilk.* N.F. **72**, 18.
Bar (1907). Leçons de pathologie obstetricale.
Birk (1910). *Monatschr. Kinderheilk.* **9**, 600.
Camerer and Söldner (1896). *Z. Biol.* **33**, 535.
Clemm (1845). Inaug. Dissertation. Gottingen.
Czerny and Keller (1925). Des Kindes, **1**, 93.
Eckles and Palmer (1916). *J. Biol. Chem.* **27**, 313.
Emmerling (1888). *Biederm. Zentr. Agriculturchem.* **17**, 861.
Engel (1911). *Ergeb. Physiol.* **11**, 73.
Guiraud (1897). Le lait de femme à l'état physiologique. Thèse de Bordeaux.
Houdet (1894). *Ann. Inst. Past.* **8**, 506.
Hunaeus (1909). *Biochem. Z.* **22**, 442.
Jansen (1918, 1). *Deutsch. Arch. klin. Med.* 125.
—— (1918, 2). *Z. physiol. Chem.* **101**, 176.
Kehrer (1920). *Archiv. Gynäk.* **112**, 487.
Lamers (1912). *Z. Geburtsh. Gynäk.* **71**, 393.
Lloyd (1926). Chemistry of the proteins, p. 46. (J. and A. Churchill, London.)
Lowenfeld, Widdows, Bond and Taylor (1927). *Biochem. J.* **21**, 1.
McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 50.
Meigs and Marsh (1913–14). *J. Biol. Chem.* **16**, 147.
Patterson (1928). *Biochem. J.* **22**, 758.
Pfaundler (1915). Doderlein's Handb. d. Geburtshilfe, p. 547.

Schlossmann (1904). *Arch. Kinderheilk.* **40**, 1.
Sebelien (1885). *Z. physiol. Chem.* **9**, 445.
Sissons and Denis (1921). *Amer. J. Dis. Child.* **21**, 389.
Söldner (1896). *Z. Biol.* **33**, 43.
Southerst (1902). *Chem. News*, **86**, 1.
Tiemann (1898). *Z. physiol. Chem.* **25**, 363.
de Wesselow (1922). *Lancet*, ii, 227.
Widdows (1923). *Biochem. J.* **17**, 34.
—— and Lowenfeld (1933). *Biochem. J.* **27**, 1400.
—— —— Bond and Taylor (1930). *Biochem. J.* **27**, 1400.
Winterstein and Strickler (1906). *Z. physiol. Chem.* **47**, 58.
Woodman and Hammond (1922). *J. Agric. Sci.* **12**, 97.
—— —— (1923). *J. Agric. Sci.* **13**, 180.

CXLV. IRRADIATION OF FATS.

I. A STANDARDISED METHOD OF USE OF ULTRA-VIOLET LIGHT.

By LESLIE HERBERT LAMPITT, NORMAN DARBY SYLVESTER AND PHILIP BILHAM.

(*From the Lyons Laboratories.*)

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During the last few years an increasing amount of work on oxidative changes in fats has been recorded, and one of the causative factors of this type of change, namely light, has received some attention. Unfortunately however very little effort has been made to study the activity of light from a quantitative standpoint. Two examples only are of sufficient importance to be noted.

Rogers and Taylor [1926] investigated the effect of light on the oxidation of linseed oil under various conditions, using a quartz mercury lamp as the source of illumination. Their statements with regard to the light energy absorbed are however based on calculations and not on direct measurements.

Read and Bailey [1933] examined the effect of ultraviolet light from a quartz mercury lamp on the development of rancidity in crackers and in shortening fats. The incident energy was measured by means of a fluorescent photometer, but no attempt was made to obtain an accurate estimate of the total light energy. Quantitative estimates of the oxidative changes were not made.

Investigations of such actions, in order to be of definite use, necessitate standard methods of evaluating the changes by chemical tests, correlation of the results obtained with observations of taste and smell if desired and a defined technique whereby figures representative of the energy of the light acting on the sample under examination can be obtained.

The work described herein deals with one of these requisites, namely the standardisation of the method of irradiation, and certain results of experiments on butter fat are recorded as examples of the applicability of the method.

Obviously it is of importance to differentiate between the total light and that which is absorbed, but no work has yet been carried out by the present authors to attempt to indicate their respective values. A complete standardisation of the conditions would involve the measurement of intensity at all wave-lengths emanating from the source of irradiation. It was found however when the absorption spectrum of butter fat in ether was determined spectroscopically (Plate III, fig. 1) that no selective absorption could be observed except that in the region 5000–4300 Å., which is due to the pigment of the butter fat, whilst the absorption spectrum (Plate III, fig. 2) of an ethereal solution of butter fat which had been irradiated until colourless showed the absence of the above band. There was however in both cases general absorption throughout the ultra-violet region of the spectrum, and it seems unnecessary therefore to exclude any wavelengths emitted by the arc in the measurement of the light intensity incident on the fat.

The statement by Gortner [1929] and attributed by him to Holm (no other reference has been found) that the greatest effect occurs when the light has a wave-length of approximately 3600 Å., is not upheld by these absorption spectra, since there is no predominating absorption at this wave-length.

It appeared therefore that the control of the total light energy of the source of irradiation was sufficient for the purposes of the present investigation.

The possibility of the occurrence of changes in distribution of the light energy emitted from an ageing lamp was not investigated, in view of the definite statement by Coblentz *et al.* [1926] that such changes do not occur. In the present experiments lamps have been used which have previously been burned for about 100 hours. It will be seen that the method of irradiation described makes allowance for any small changes which may occur in the quantity of light emitted from the lamp.

EXPERIMENTAL.

(*a*) *Electrical equipment.*

After several abortive attempts to obtain standard and reproducible experimental conditions, the lay-out of apparatus shown in Fig. 1 was finally evolved.

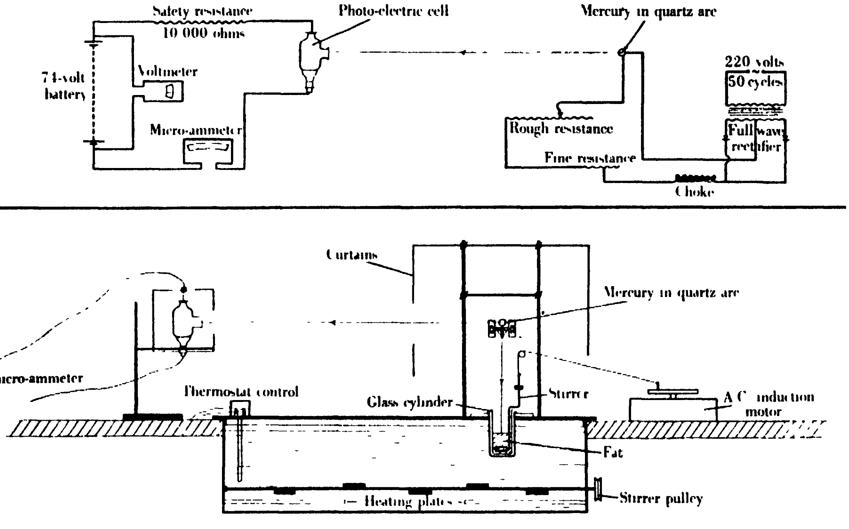

Fig. 1.

The fat to be irradiated was contained in a cylindrical glass vessel which fitted into a metal holder, supported from the cover of a thermostatically controlled water-bath. The mercury quartz arc lamp (Kelvin, Bottomley and Baird) was clamped in a fixed position, the centre of the arc being vertically above the tube containing the fat.

A measure of the light intensity of the lamp was obtained by means of a photo-electric cell (U.N.G. 7 type G.E.C.) having an activated sodium cathode in a glass bulb with a quartz window. A constant potential of 74 v. was applied to the cell from accumulators[1], and the photo-current was measured by means of a micro-ammeter.

The current for the lamp was supplied by a rectifier from the A.C. mains. Two variable resistances were placed in series with the lamp, and the major

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[1] In earlier experiments the cell potential was applied from the A.C. mains through a rectifier; difficulties due to fluctuation of the mains potential however made this method impracticable.

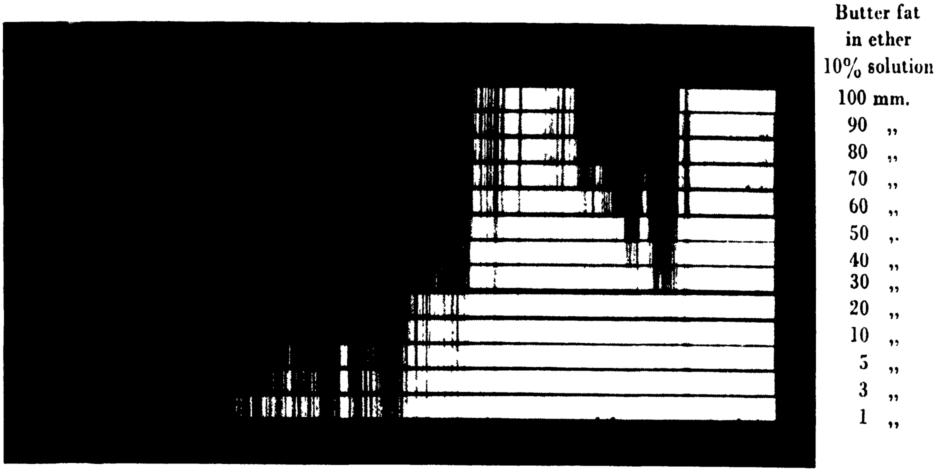

Fig. 1. Absorption spectrum of fresh butter fat in ether.

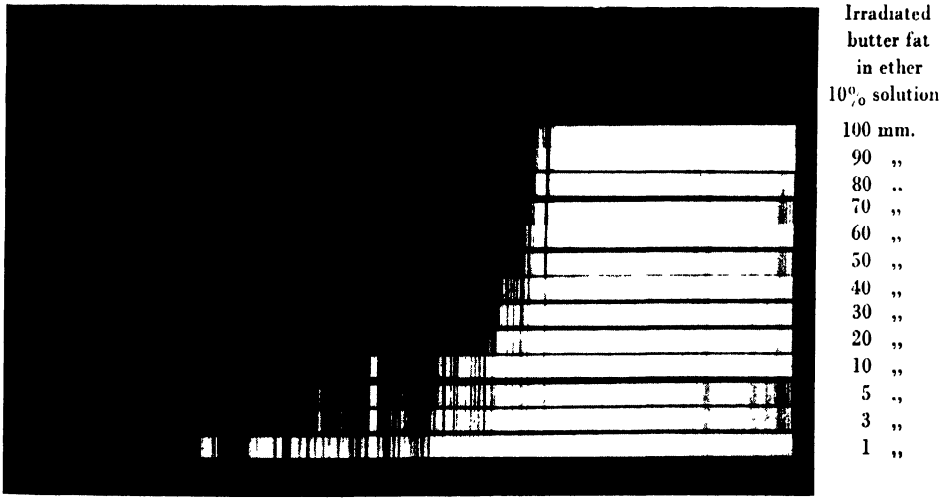

Fig 2. Absorption spectrum of irradiated butter fat in ether.

alterations in intensity were effected by means of the "rough" resistance, the light from the lamp being approximately adjusted to any required intensity (as indicated by the micro-ammeter reading), accurate adjustments being made with the "fine" resistance.

During the progress of irradiation it was necessary to make periodic adjustments to the fine resistance in order to restore the micro-ammeter reading to the required magnitude. These corrections were necessitated by fluctuations in the A.C. voltage, and they were required at all times of the day and night. Fortunately the major fluctuations occurred at fairly definite times and could be anticipated.

The fat was stirred with an up-and-down motion by means of a glass rod stirrer, shown in Fig. 2, which was designed so that the obstruction of the light would be at a minimum. The stirrer was guided by movement through a vertical glass tube, and was actuated by an A.C. induction motor, the speed of which varied between 63 and 68 revolutions per minute.

Irradiation experiments could then be carried out at any fixed light intensity which was indicated by the micro-ammeter reading, and the effect of slight variations in the A.C. supply, as well as that due to ageing of the lamp, were corrected by periodic adjustments to the resistance.

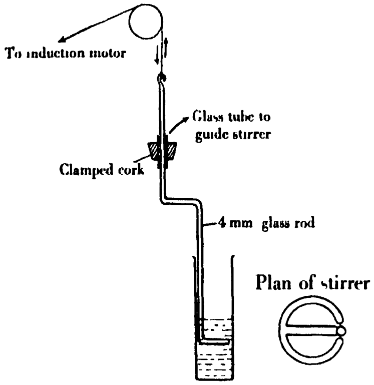

Fig. 2. Glass stirrer used in irradiation apparatus.

(b) Measurement of the light intensity in absolute units.

In order that the micro-ammeter readings obtained with the cell in the remote position might be related to the actual light energy falling on the surface of the fat, it was necessary to determine a conversion factor. The cell responded only to a limited wave-band and therefore the standardisation of the cell against a standard of radiation was made through the medium of a thermopile.

Calibration of the thermopile. The thermopile was used, without its collecting funnel, in conjunction with a micro-ammeter. The calibration of this combination was made with a carbon lamp, standardised by the National Physical Laboratory and used, under the conditions specified in their certificate, as the source of radiation. The response obtained was 0·155 micro-amp. for an incident radiation of $5{\cdot}864 \times 10^3$ ergs per second per cm.^{2} A reading of 1·0 micro-amp. would therefore be equivalent to $3{\cdot}78 \times 10^4$ ergs per second per cm.^{2} radiation on the surface of the thermopile.

This figure was roughly checked by the use of a Leslie cube. The intensity of the radiation R on a spherical wave front from the black face of the cube is given by the formula:

$$R = \frac{\sigma (T^4 - t^4) \times E \times A}{2\pi r^2} \text{ ergs per second per cm.}^2,$$

where σ = Stefan's constant $= 5{\cdot}72 \times 10^{-5}$,
T = temperature of cube = 95°,
t = temperature of air = 19·5°,
E = efficiency of radiation from cube = 0·9 [Poynting and Thomson, 1919],
A = area of black face = 20·5 cm. × 20·5 cm.,
r = radius of spherical wave front = 50 cm.

When the values were as stated above the response given by the thermopile was 0·34 micro-amp. It may be calculated from these figures that the incident radiation for a response of 1·0 micro-amp. would have been 4·45 × 10^4 ergs per second per cm.^{2}

This figure is subject to some inaccuracy, due to departure from the inverse square law, owing to the extended surface of the source as compared with its distance from the thermopile. It served as a check on the first figure which has been used in all subsequent calculations.

The conversion factor can be determined in two ways:

1. *The standardisation of the cell against the calibrated thermopile and the measurement of the ratio of the micro-ammeter reading to a hypothetical reading which would be obtained if the cell occupied the position of the irradiated fat.*

The comparison of the responses of the calibrated thermopile and the cell to the light of the arc was carried out by rapidly substituting one instrument for the other when the arc was burning steadily, the surface of the thermopile occupying the same position as the outer surface of the quartz window of the cell. The responses obtained were 3·04 micro-amps. and 1·01 micro-amps. for cell and thermopile respectively and therefore the radiation from the arc which would give a reading of 1·0 micro-amp. when incident on the window of the cell would be 1·25 × 10^4 ergs per second per cm.^{2}

The ratio of the actual to the hypothetical micro-ammeter readings was determined by direct experiment. When the outer surface of the cell window occupied the same position as the fat surface, the light was so intense that the cell would have given a current far in excess of its normal working range and not proportional to the light intensity. To avoid this error the incident light was reduced by a non-selective filter consisting of a tube on each end of which was tightly stretched and soldered fine mesh wire gauze. Two such filters were used having transmissions of 6·25 and 12·2 %, which factors, combined with the responses, gave values of 8·0 and 7·65 for the ratio. The mean value of 7·8 has been taken.

Using the figures obtained in the above experiments, it is possible to calculate the energy per second incident on the fat surface of area 9·2 cm.^{2} when a reading of 1·0 micro-amp. is given by the cell in the remote position. The result is *0·090 ×* 10^7 *ergs per second.*

2. *The measurement of the energy at the fat surface with the calibrated thermopile and the correlation of this with the response of the cell in the remote position.*

When the cell in the remote position was giving a response of 3·9 micro-amps., the thermopile was placed so that its surface occupied the same plane as that normally occupied by the fat and gave a response of 8·6 micro-amps. The light energy per second incident on the surface of the fat when the cell is giving a reading of 1·0 micro-amp. in the remote position can be calculated from the previous calibration of the thermopile. The result is *0·077 ×* 10^7 *ergs per second.*

Since neither method can be considered the more accurate, the mean of the two results, *viz. 0·084 ×* 10^7 *ergs per second,* has been used for calculating the total incident light energy in the irradiation experiments to be described.

(c) *Standard procedure.*

The following are the summarised conditions of standard experiments:

Distance, centre of arc to surface of fat	25·5 cm.
Area of fat surface exposed	9·2 cm.^{2}
Weight of fat	50 g.
Depth of fat	6·0 cm.
Distance of photo-electric cell from arc	91·5 cm.

Under these conditions the effects of the irradiation of the butter fat were reproducible, and the chemical determinations satisfactory, even when duplicate experiments were carried out after an interval of as much as 14 days.

Results obtained using the standard irradiation apparatus.

The experiments, the results of which are given in subsequent tables, were carried out on pure butter fat, prepared from best quality Australian unsalted butter. The fat was separated after melting the butter at a low temperature, filtered twice through filter-paper and stored in vacuum jars in the dark.

A point of interest is concerned with the amount of fat taken. Since the extent of oxidation of the fat is not proportional to the time of irradiation, it follows that if, for example, 25 g. of butter fat were taken, the oxidation would not be twice that observed in the normal experiment using 50 g. of fat. It was decided however to carry out experiments in which the weight of fat was reduced to 25 g., the period of irradiation being also reduced to one-half. The other experimental conditions such as area of fat exposed, distance from the lamp, temperature and intensity of the incident light were the same for both experiments.

The results obtained are given in Table I.

Table I.

Time of irradiation	15 hours	30 hours
Weight of fat	25 g.	50 g.
Issoglio value	41·3	41·4

The above results were confirmed by duplicate experiments. It appears that the oxidative changes in the fat are not appreciably affected by a variation in the amount of fat taken, provided that the time of irradiation is proportionately altered. It is obvious that the depth of the fat is an important factor, and that the above conclusion will not be true when the depth of the fat is so decreased that an appreciable proportion of the active light is transmitted by the fat without exercising any photochemical effect.

Irradiations were made under various conditions of time, temperature and light intensity. The other experimental conditions were those which have already been described.

Table II. *Effect of light intensity.*

Time of irradiation constant at 20 hours. Temperature 40°.

Micro-ammeter reading	—	1·5	2·0	3·0	3·5	4·0
Watt-secs.	Nil	9,000	12,000	18,000	21,000	24,000
Peroxide value	1·3	3·8 3·7	4·3	6·2 5·8	8·6	12·5 13·9
Issoglio value	2·1	1·95 2·05	6·0	13·3 12·1	15·6	20·7 19·6
Kreis test	Neg.	4 4	10	35	70	100 100

Table III. *Effect of time.*

Light intensity constant at 2·75 watts per cm.^{2} (micro-ammeter reading 3·0). Temperature 40°.

Irradiation time	Nil	10 hrs.	20 hrs.	30 hrs.	40 hrs.	50 hrs.	70 hrs.
Watt-secs.	Nil	9,000	18,000	27,000	36,000	45,000	63,000
Peroxide value	0·8	3·5 3·1	5·2 4·9	32·0 26·5	45·5 47·5	68	120
Issoglio value	1·9	2·7 2·4	10·2 10·5	32·0 31·0	—	—	116
Kreis test	Neg.	5 5	18 20	200 200	650	1300	2650

Table IV. *Effect of time.*

Light intensity constant at 2·75 watts per cm.^{2} (micro-ammeter reading 3·0).
Temperature 60°.

Irradiation time	Nil	10 hrs.	20 hrs.	25 hrs.	30 hrs.	40 hrs.	50 hrs.
Watt-secs.	Nil	9000	18,000	22,500	27,000	36,000	45,000
Peroxide value	0·8	2·4	8·0 8·6	23·5 23·0	39·0 46·5	78	117
Issoglio value	1·9	3·4	19·0 19·8	30·0 30·5	47·0 46·0	83	—
Kreis test	Neg.	4	50 50	170	650	2000	4000

In the above tables where two sets of figures occur in one column, they represent the results of duplicate irradiations carried out in some cases after an interval of 2 weeks. The duplication of the results is seen to be satisfactory.

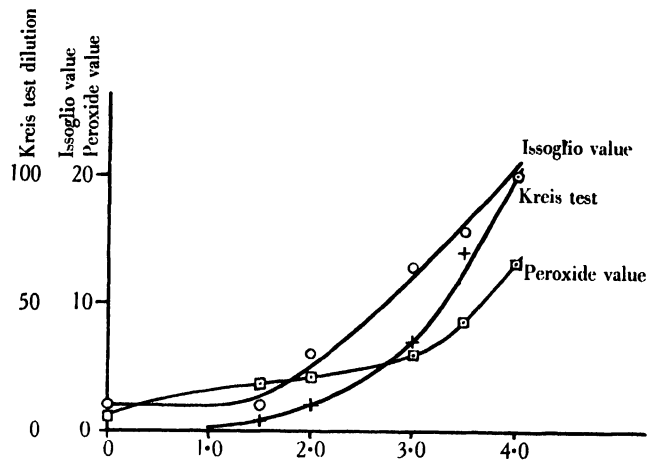

Fig. 3. Effect of light intensity.

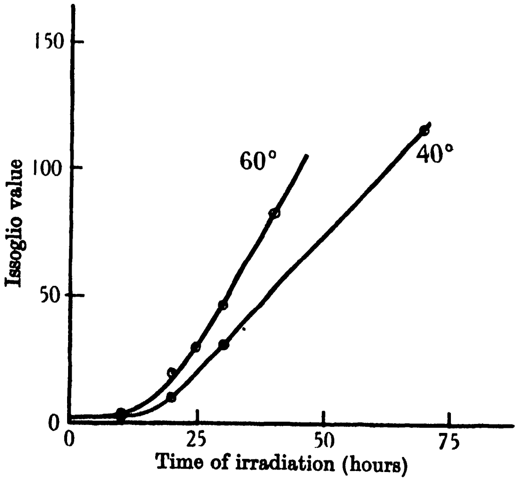

Fig. 4. Effect of time and temperature on Issoglio value.

Peroxide values, and Issoglio values are both expressed as mg. O_2 per 100 g. fat, and the Kreis test is expressed as the first dilution (in light petroleum) at which a positive test is not obtained. The methods of analysis will form the subject of a separate paper.

The results are shown graphically in Figs. 3, 4, 5 and 6.

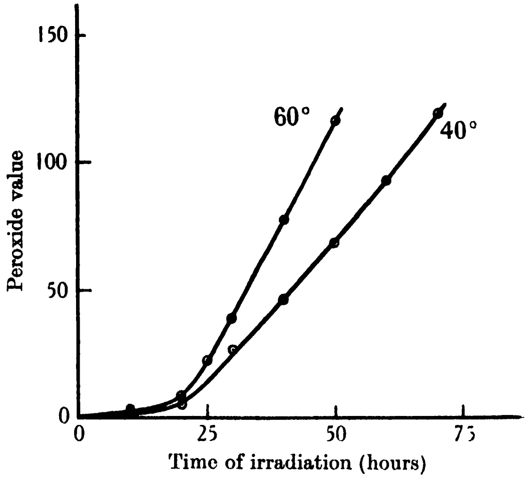

Fig. 5. Effect of time and temperature on peroxide value.

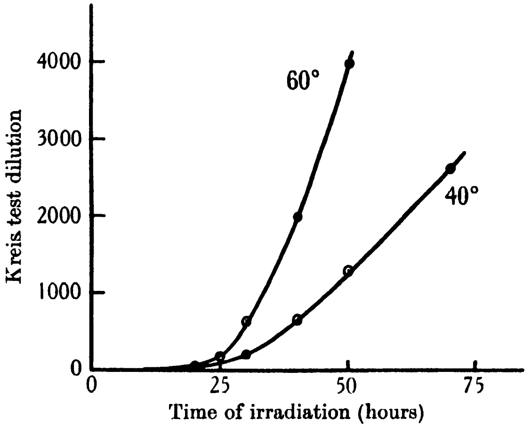

Fig. 6. Effect of time and temperature on Kreis test.

Discussion.

The utility of such an apparatus as has been described is obviously not restricted to the type of experiment described in this paper, and this method of irradiation has, for example, been used by the authors in an investigation on the development of tallowiness in milk. Moreover, it could be applied to determinations of the induction period of a fat with a view to assessing its potential keeping properties, and such factors as the addition of pro- and anti-oxidants, variations in methods of fat processing *etc.* could be examined with regard to their effects on the susceptibility of the fat to oxidative deterioration. Such determinations can be made with the certainty that the experimental conditions can be reproduced and the results confirmed.

The results which have been given on the irradiation of butter fat have been included principally to illustrate the utility of the technique which has been described, and they are not presented as a definite contribution to the subject of oxidative changes in fats.

A consideration of the figures will show, however, that although the three determinations increase concurrently, and in a similar manner, there is not a strict parallelism between any two of them.

The effect of temperature is clearly demonstrated and has an effect on the magnitude of all the determinations. It is seen further that any relationship which might exist, say, between the Kreis test and the Issoglio value is changed by temperature. Lea [1931] has made a similar observation with regard to the Kreis test and peroxide value and the present results afford confirmation of his assertion.

SUMMARY.

1. An irradiation technique has been worked out suitable for experiments on oils and other liquids, such that good reproducibility of results can be obtained.
2. A method has been indicated whereby measurements made in a position other than that of the sample have been correlated with the intensity, in absolute units, of the light incident on the fat.
3. Butter fat has been irradiated under standardised and reproducible conditions. The oxidative changes have been measured by chemical tests, and an approximate determination of the incident light energy has been made.

The authors wish to thank Messrs J. Lyons and Co., Ltd., in whose laboratories this work was conducted, for permission to publish this paper.

REFERENCES.

Coblentz, Dorcas and Hughes (1926). *U.S. Bureau of Standards Scientific Paper*, No. 539, p. 545.

Gortner (1929). Outlines of biochemistry, p. 641. (John Wiley and Sons, New York.)

Lea (1931). *Proc. Roy. Soc. Lond.* B **108**, 175.

Poynting and Thompson (1919). Heat (5th edition), p. 321. (Griffin, London.)

Read and Bailey (1933). *Cereal Chem.* **10**, 99.

Rogers and Taylor (1926). *J. Physical Chem.* **30**, 1334.

CXLVI. THE BEHAVIOUR OF PATHOLOGICAL SERA IN THE ULTRACENTRIFUGE.

By ARTHUR SPROUL McFARLANE[1].

From the Institute of Physical Chemistry, Upsala, Sweden.

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In a previous paper [McFarlane, 1935, 1] we have described the results of the examination of horse serum albumin and globulin in the ultracentrifuge, both separately and in the form of mixtures of the two. It was shown that these two proteins behave ideally in dilute mixtures of the two, but in concentrated mixtures their general behaviour is abnormal. In particular, each molecular fraction is present in abnormal concentration, there being always more of the lighter ("albumin") fraction present than corresponds to the albumin added and correspondingly less of the heavier ("globulin") fraction. It is believed that there exists in mixtures of serum albumin and globulin a form of molecular dimensional equilibrium the exact nature of which has still to be discovered. In the course of this work a modified optical system for the ultracentrifuge has been developed which enables protein solutions as highly concentrated as untreated serum to be examined.

In a second paper [McFarlane, 1935, 2] we have described the application of this system in the analysis of the serum proteins of the normal cow, horse and man. Since it is clearly a matter of great interest to know the molecular dimensional distribution of the protein in untreated serum it was our aim to examine these sera as far as possible without dilution. In the case of human serum, however, it is necessary to dilute slightly in order to avoid a form of distortion in the photographs, the nature and cause of which is discussed. It has been shown that this slight dilution of a normal human serum does not affect the results, whereas dilution of all sera with more than 2–3 vols. of 1 % sodium chloride causes an alteration in the concentration of the fractions which is in exact agreement with the facts relating to artificial mixtures of albumin and globulin. We have found that the sera of all three species are constituted in a similar manner of three molecular fractions, two of which correspond approximately in sedimentation constants (with due allowance for the effect of protein concentration) to purified horse serum albumin and globulin. The chemical nature of the third fraction, of molecular weight slightly greater than albumin, is obscure and it is not proved that the substance is capable of an independent existence. It appears from the examination of sera from a series of normal healthy persons of various ages and of both sexes that the concentration ratios of the three fractions vary only within narrow limits in the presence of large variations in the concentration of total protein. The concentration of the globulin fraction in the series is (10 ± 5) % of the total protein.

In the present paper there are presented in detail the results of the examination of a series of pathological sera, chosen more or less at random, and obtained mostly from the general hospital in Upsala[1]. We wish to emphasise the general nature of the investigation. There has been repeatedly in the course of these experiments a strong temptation to interrupt the series in order to pursue in greater detail the investigation of one or two particularly interesting sera. We resisted this because it was believed that a more useful purpose could be served at the moment by collecting a series of results which would give an impression—however superficial—of the field as a whole. This is our justification for omitting to proceed with several more detailed investigations which logical consideration will at once suggest to the reader might have been done on certain sera. We need hardly say that it is planned to investigate many of these individual problems in the immediate future.

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[1] Beit Memorial Research Fellow.

In the two previous papers [1935, 1, 2] there will be found a complete description of the general technique employed in this work. We will only draw attention here to one important fact, *viz.* that all calculations of protein concentration are based on the apparent baseline of the sedimentation diagram. As a result the only method of detecting the presence of any polydisperse, and presumably denatured, protein is by comparing the protein refraction increment of the serum, as measured with the refractometer, with the total refraction increment from the area of the sedimentation curve. We have, therefore, in most cases, dialysed a portion of the serum at constant volume against 1 % sodium chloride and measured the refractive indices of solution and dialysate using the difference prism of the Pulfrich refractometer and the sodium D line. In certain cases of nephritis the serum was characteristically turbid owing to suspended lipoid matter and considerable difficulty was experienced in detecting any transmitted beam in the refractometer. Centrifuging for an hour or more at 3000 r.p.m. in a standard laboratory centrifuge failed to produce any detectable reduction in the degree of turbidity. The effect of this turbidity in the centrifuge-cell as seen from the camera is to give rise to a green coloration due to the selective scattering of the lower wave-lengths in the mercury spectrum. It is interesting to record in these cases that as the ultracentrifuge accelerated a band of clear serum appeared at the bottom of the cell and the green coloration invariably disappeared into the region of the meniscus within 10–15 minutes from starting, and before the rotor had attained a speed of 25,000 r.p.m.

The conditions of the various experiments have been made more comparable by employing as far as possible centrifuge-cells 2·0 mm. in thickness and by using centrifugal forces uniformly in the region of 250,000 times gravity.

Each experiment is recorded under the initials of the donor of the serum and the clinical diagnosis of the condition. In the form of an appendix there is given a series of notes on each case, those features in the history being stressed which might have a connection with the state of the serum proteins.

In accordance with previous practice we shall refer to the fractions in a serum as A and G which correspond in sedimentation constants to purified horse serum albumin and globulin, and to other fractions as X, Y, Z, in order of increasing sedimentation constant.

In order to determine whether two fractions correspond in sedimentation constants it is necessary to take into consideration the concentration in which the fractions are present. This should be possible in the case of the lightest fractions with considerable accuracy since the boundary is sedimenting in a medium which is free of other proteins. In the case of the heavier fractions however, this is not so, and it is not possible with the data at present available to effect more than an approximate comparison of the sedimentation constants of corresponding *X* or *G* fractions. In order to facilitate the identification of *A* fractions we have constructed the graph in Fig. 1 by plotting the values already found for the *A* boundary sedimentation constants of normal human sera, and shown in Table XVII of a previous paper [1935, 2], against the refraction increments $(n_1 - n_0)$ of the same fractions. We shall have occasion to refer to it in the description of the following experiments.

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[1] I am greatly indebted for facilities to obtain pathological sera to Profs. G. Bergmark, G. Nyström, and Axel Westman of Kungl. Akademiska Sjukhuset, and to Dr O. Bratt of Epidemisjukhuset, Upsala.

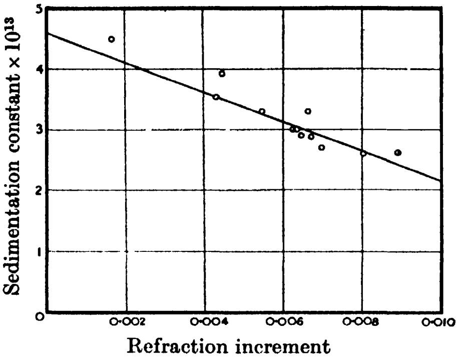

Fig. 1. Showing the relationship between the refraction increments and sedimentation constants of the *A* fractions of normal human sera. Data taken from Table XVII of earlier paper [1935, 2].

In the preceding paper [1935, 2] we have calculated the concentrations of the protein fractions in g./100 ml. from the specific refraction increment (α) values of the albumins and globulins described in the literature. In this work we shall calculate only the total protein concentrations using the values α (total horse serum) = 0·00184, and α (total human serum) = 0·00202, both for the sodium *D* line.

Before describing the experiments with pathological human sera there will be given some results obtained with two samples of antidiphtheritic horse serum kindly supplied by Messrs Burroughs and Wellcome, Beckenham, Kent.

Exp. 1. Antidiphtheritic horse serum.

Serum 1, containing 200 units of antitoxin, was examined undiluted in a cell 2·00 mm. in thickness, and excellent scale photographs were obtained with the scale 1·0 cm. from the centre of the cell. When allowance is made for the refractive index of the quartz cell plate this distance corresponds to an optical distance of 0·78 cm. The experimental conditions and results are shown in Table I, and Fig. 2 *a* shows the curve of an exposure taken 83 minutes after reaching full speed. The distance separating the two boundaries is small, but owing to the small amount of boundary spreading in this concentrated mixture, it is sufficient to give a symmetrical graphical delineation of both boundary curves. The sedimentation constant of the *G* fraction is unusually low. Owing to the high concentration of this fraction, however, and to the fact that the *G* molecules are sedimenting in a dense and viscous medium of *A* molecules, the effect of which is not taken into account in the $S_{W,20}$ figure, the value of the constant is not inconsistent with the fraction having a molecular weight the same as that of horse serum globulin. We can find no evidence of an X fraction such as is present in normal horse serum, but this may be present in low concentration and not detectable because of the overlapping of the two boundaries.

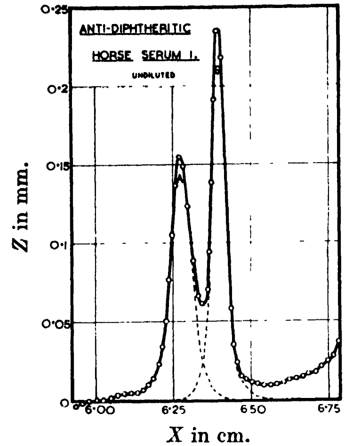

Fig. 2a

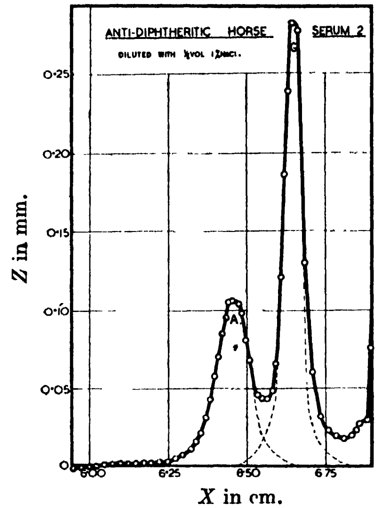

Fig. 2b.

Table I. *Results obtained in the examination of two antidiphtheritic horse sera.*

		Serum 1. Undiluted	Serum 2. Undiluted	Serum 2. Diluted with ½ vol. 1 % NaCl
Cell thickness (mm.)		2·00	1·50	2·00
Optical scale distance (cm.)		0·78	1·12	1·69
Temperature inside cell at time of first exposure		36·3°	33·9°	33·4°
Temperature inside cell at time of last exposure		38·6°	36·1°	35·0°
Approximate duration of run (hours)		2½	2½	2
Mean centrifugal force used (times gravity)		245,000	255,000	250,000
Total $n_1 - n_0$, by refractometric measurement on the serum and its dialysate; $\lambda = 589\ m\mu$		0·0204	0·0177	0·0118
Corresponding total protein concentration (g./100 ml.)		11·1	9·62	6·41
Total $n_1 - n_0$, corrected to $\lambda = 366\ m\mu$		0·0220	0·0191	0·0127
Average $S_{W,20} \cdot 10^{13}$	A fraction	2·97	3·00	3·86
	G fraction	3·71	3·86	5·11
Average $n_1 - n_0$, calculated from sedimentation diagram, $\lambda = 366\ m\mu$	A fraction	0·0102	0·0090	0·0052
	G fraction	0·0109	0·0089	0·0073
	Total	0·0211	0·0179	0·0125
Difference between measured and calculated total $n_1 - n_0$ expressed as a percentage of the former		-4 %	-6 %	-2 %
Proportion of total calculated $n_1 - n_0$ due to G fraction		52 %	50 %	58 %
Spreading coefficient of A boundary (cm.²/sec. × 10⁷)		0·4	0·3	3·8

From our experiences with normal sera we are of opinion that only very small amounts of X fraction, if any, can be present. The value of the spreading coefficient of the A boundary is in favour of this fraction being homogeneous.

Serum 2, containing 1200 units of antitoxin, was also examined without treatment of any kind and gave rise to sedimentation diagrams very similar to those of serum 1. The results of this experiment are shown in Table I and it will be seen that they are generally similar to the results of the previous experiment. In particular, it is noteworthy that there is no increase in the proportion of *G* fraction which might correspond to the increased antitoxic value of serum 2. We have also examined the same serum after slight dilution with 1 % sodium chloride. Fig. 2 *b* shows the curve of an exposure from this experiment taken 81 minutes after reaching full speed. It will be seen from this and from the results in Table I that there has occurred a considerable increase in the concentration of the *G* fraction, and a proportionate decrease in that of the *A* fraction. The extent of separation of the two boundaries has not been greatly affected by the dilution although the sedimentation constants of both the *A* and *G* fractions are quite markedly increased. There is also a substantial increase in the degree of spreading at the *A* boundary.

In all three experiments there is very close agreement between the measured and calculated total protein refraction increment values. This is probably accidental since with very short scale distances the experimental error is increased considerably.

Exp. 2. Serum from A.J. (ovarian carcinoma).

This serum from an advanced case of malignancy was examined first without any treatment and then after dilution. Large quantities of pleural exudate were also available which resembled the serum closely in colour and general appearance. A sample of this was examined also without treatment of any kind. The results of all three experiments are collected in Table II, and Fig. 3 *a* shows the curve of an exposure in the first experiment taken 112½ minutes after reaching full speed. Comparison of this curve with that on Fig. 14 of the previous paper [1935, 2] showing a typical normal human serum, shows that gross changes have taken place.

In this malignant serum three fractions are present which we have labelled *A*, *G* and *X*. The sedimentation constant of the lightest fraction, *viz.* 2·32, is considerably lower than the corresponding value for the *A* fraction of a normal human serum obtained from Fig. 1, *viz.* 3·4. The difference between these values is much greater than could be explained by any experimental error. The value for the *A* fraction in Table II is indeed lower than any value for an *A* fraction recorded in Table XVII of the previous paper irrespective of concentration. The evidence that we are dealing with a substance of lower molecular weight than serum albumin seems therefore to be quite definite. It is to be noted however from Table II that the sedimentation constants of the *X* and *G* fractions in this serum are unusually low, and also that the sedimentation constants of the three fractions bear approximately the same ratios to each other as do those of the *A*, *G* and *X* fractions in normal human sera. These facts suggest either that all the fractions have abnormally low molecular weights or else that some common factor is acting on normal *A*, *G* and *X* fractions to make them sediment abnormally slowly.

There is no evidence in the large number of experiments carried out by Svedberg and collaborators on purified proteins of the existence of any such obscure influence. We have noted, however, that in their experiments a better degree of agreement is to be found between the values for the sedimentation constant of a particular protein than we have obtained between values for the *A* fraction of normal human sera (*cf.* Fig. 1). In whole serum it is possible that some factor, perhaps electrochemical in nature, or some substance, possibly lipoid, by influencing the molecular shape or charge, or the viscosity at the surface of the particles, may cause a change in the sedimentation constant without altering the molecular weight. These possibilities cannot be excluded so long as we are examining a solution of relatively undefined composition. So long as they exist an element of doubt attaches to our method of identifying the serum fractions, particularly in cases where all the fractions have abnormal sedimentation constants. It cannot therefore be said without further investigation whether any or all of the fractions in the serum of A.J. differ in molecular weight from the *A*, *G* and *X* fractions of normal sera. As a matter of convenience we have labelled these fractions meantime as *A*, *G* and *X*.

The proportions of both *X* and *G* fractions in this serum are abnormally high. This is perhaps best brought out by comparing the average proportion of *A* fractions in normal human serum, 66 %, with the proportion in this case,

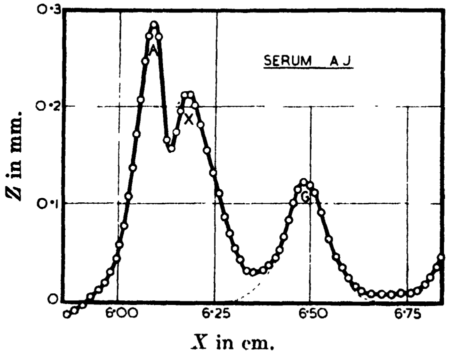

Fig. 3*a*.

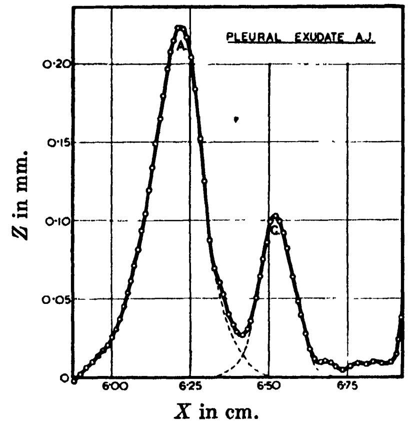

Fig. 3*b*.

Table II. *Results obtained in the examination of serum and pleural exudate from A.J. (ovarian carcinoma).*

		Serum A.J. Undiluted	Serum A.J. Diluted with 4 vols. 1 % NaCl	Pleural exudate A.J.
Cell thickness (mm.)		2·00	4·00	2·00
Optical scale distance (cm.)		3·64	9·64	5·14
Cell temperature (first exposure)		31·9°	30·6°	31·6°
Cell temperature (last exposure)		34·5°	32·9°	34·1°
Approximate duration of run (hours)		$3\frac{1}{4}$	2	$2\frac{1}{4}$
Mean centrifugal force used (times gravity)		245,000	240,000	245,000
Total $n_1 - n_0$, by refractometer $\lambda = 589\ m\mu$		0·0128	0·00256	0·00745
Corresponding total protein concentration (g./100 ml.)		6·34	1·27	3·69
Total $n_1 - n_0$, corrected to $\lambda = 366\ m\mu$		0·0138	0·00276	0·00805
Average $S_{W,20} \cdot 10^{13}$	*A* fraction	2·32	4·66	3·51
	G fraction	4·20	6·93	5·73
	X fraction	2·73	—	—
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\ m\mu$	*A* fraction	0·0050	0·00146	0·0056
	G fraction	0·0031	0·00093	0·0021
	X fraction	0·0036	—	—
	Total	0·0117	0·00239	0·0077
"% difference" total $n_1 - n_0$		-14 %	-13 %	-4 %
Proportion of total $n_1 - n_0$ due to *G* fraction		26·5 %	39 %	27 %
Proportion of total $n_1 - n_0$ due to *X* fraction		31 %	—	—
Spreading coefficient of *A* boundary (cm.²/sec. 10⁷)		—	21·3	16·1

42·5 %. It is not possible to effect a symmetrical graphical separation of the overlapping *A* and *X* curves in this serum for reasons discussed in the previous paper, so that the *A* and *X* concentration values given are not exact. The combined $A+X$ value is exact, and in our graphical estimation of the *A* and *X* concentrations in the present case we have given the benefit of the doubt to the *A* fraction. The value for the *A* fraction can therefore be regarded as an upper limit to the true value. Similarly, the true *X* value is not less than the one given. Also, owing to the extent of overlapping of the *A* and *X* boundaries we cannot estimate the spreading coefficient of the *A* boundary.

In the second experiment the serum was diluted with 4 volumes of 1 % sodium chloride. The sedimentation rates of both boundaries are markedly increased. The degree of *A* boundary spreading is also much increased and definite signs of a separation of the lighter fraction into *A* and *X* fractions cannot consequently be seen. The curves of the more slowly sedimenting boundary are however all unsymmetrical in a manner suggesting the presence of two fractions in concentrations not differing greatly from each other. The heterogeneity of the fraction is confirmed by the general dimensions of the curve and the extremely high value of the spreading coefficient, *viz.* 21×10^{-7} cm.^{2}/sec. In Table IV of a previous paper [1935, 1, p. 423] we have shown the spreading coefficient values for a boundary of recrystallised horse serum albumin (sedimenting under standardised centrifuge conditions) plotted against the protein refraction increment of the solution. From this curve we find that a sedimenting protein boundary, assumed to be homogeneous, of the same protein concentration as the *A* boundary in this experiment has a spreading coefficient of approximately $6{\cdot}0 \times 10^{-7}$ cm.^{2}/sec.

Table II also shows that there has been a definite increase in the proportion of the total protein refractivity due to the *G* fraction on diluting the serum.

In the third experiment we examined the untreated pleural exudate. Fig 3 *b* shows the curve of an exposure taken 83 minutes after reaching full speed. The results of this experiment are also shown in Table II. It happened that the total protein concentration in the pleural exudate was almost an exact mean between the corresponding values in the two preceding experiments. Table II shows that the sedimentation constants of the two fractions in the pleural exudate are also roughly a mean between the values in the former experiments. From this we deduce that the protein fractions in the pleural exudate have approximately the same mean molecular dimensions as the corresponding protein fractions in the serum.

From Fig. 3 *b* it will be seen that the *A* curve does not show any obvious signs of dividing into two components, but it does not have the perfect symmetry which has characterised the homogeneous proteins in our earlier experiments [1935, 1]. Once again, the view that we are concerned with a mixed fraction, the heterogeneous nature of which is obscured by the increased spreading of the boundaries, is confirmed by the general dimensions of the curve and by the high value of the spreading coefficient, *viz.* 16×10^{-7} cm.^{2}/sec. The value for a purified albumin boundary of corresponding protein concentration is $1{\cdot}6 \times 10^{-7}$ cm.^{2}/sec. The proportion of *G* fraction in the pleural exudate is in agreement with the other results and all conform with the view that the fluid is a passive transudate of whole serum which has undergone simple dilution in passing from the blood into the pleural cavity.

Exp. 3. Oxalate plasma from K.H.B. (acute phthisis).

In this case we obtained oxalate plasma and no preliminary dialysis was carried out. The plasma did not keep well over the period of the experiments but gave rise to a small deposit of fibrin. The amount of protein thus removed from the solution was not sufficient to affect materially the results obtained. In the first experiment we examined the undiluted plasma and obtained curves showing marked distortion of the A and X curves. The G curve was symmetrical, however, and from the area of this curve we find:

Calculated $n_1 - n_0$ of G fraction in undiluted plasma $= 0{\cdot}00318$.

The plasma was then examined after dilution with one-third its volume of 1 % sodium chloride. Fig. 4 *a* shows the curve of an exposure taken 98½ minutes after reaching full speed, and the general results of the experiment are recorded in Table III.

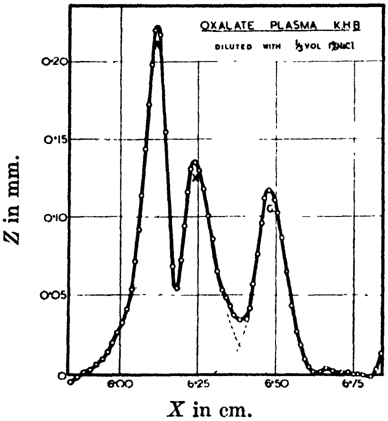

Fig. 4*a*.

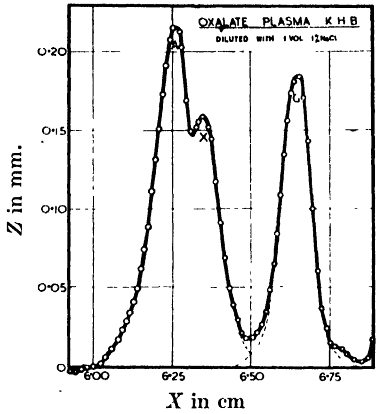

Fig. 4*b*.

Table III. *Showing the results obtained in the examination of oxalate plasma from K.H.B. (pulmonary tuberculosis).*

		Plasma diluted with ⅓ vol. 1 % NaCl	Plasma diluted with 1 vol. 1 % NaCl	Plasma diluted with 2 vols. 1 % NaCl
Cell thickness (mm.)		2·00	2·00	2·00
Optical scale distance (cm.)		2·74	6·14	6·14
Cell temperature (first exposure)		34·4°	32·8°	33·3°
Cell temperature (last exposure)		35·2°	34·1°	35·3°
Approximate duration of run (hours)		3½	2½	2½
Mean centrifugal force used (times gravity)		250,000	245,000	245,000
Average $S_{W,20} \cdot 10^{13}$	A fraction	2·79	3·23	4·17
	G fraction	4·72	5·40	6·22
	X fraction	3·12	4·42	—
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\ m\mu$	A fraction	0·00454	0·00461	0·00274
	G fraction	0·00324	0·00260	0·00219
	X fraction	0·00338	—	—
	Total	0·01116	0·00721	0·00493
Calculated total $n_1 - n_0$ of original undiluted serum		0·0149	0·0142	0·0148
Proportion of total $n_1 - n_0$ due to G fraction		29 %	36 %	44 %
Proportion of total $n_1 - n_0$ due to X fraction		30 %	—	—

A comparison of the curve with that of Fig. 3 *a* shows a close general similarity to exist between the two. The sedimentation constant of the lightest fraction, 2·79, is considerably lower than the value 3·5 for a normal *A* fraction in corresponding concentration (*cf.* Fig. 1). As in the previous experiment however the $S_{W,20}$ values for all the fractions are unusually low and bear approximately the same ratios to each other as do the values for the fractions of normal sera. We conclude that this plasma also represents a case in which it cannot be said without further investigation whether the fractions are identical with the fractions of normal sera.

The calculated value of $n_1 - n_0$ for the *G* fraction is interesting. If this value is corrected for the dilution we find:

Calculated $n_1 - n_0$ of *G* fraction in original plasma based on proportion found after dilution ... ... 0·00432

We conclude that unlike the state of affairs in a normal human serum the effect of even slight dilution of a pathological serum is to produce a change in the concentration ratio of the fractions which is apparent as an increase in the absolute concentration of the *G* fraction. We have studied the effect of dilution further in this case by examining the plasma after dilution with an equal volume, and with twice its volume, of 1 % sodium chloride. The results of these experiments are collected in Table III and call for little comment. It is apparent that the proportion of the *G* fraction increases steadily on dilution. We cannot say whether there is at the same time any change in the ratio of the *A* and *X* fractions because the two do not separate sufficiently in the more dilute solutions. Fig. 4 *b* shows the curve of an exposure taken 97½ minutes after reaching full speed in the experiment with plasma diluted with an equal volume of 1 % sodium chloride. The dual nature of the lighter boundary is rather obvious but we cannot resolve the curve graphically. In the final experiment the *A* curve has only one peak but it is unsymmetrical, with a squat appearance (*i.e.* a high ratio of breadth at the base to height) and an abnormally high spreading coefficient, *viz.* 9×10^{-7} cm.^{2}/sec.

Exp. 4. Sera from B.I.L. and J.O.D. (scarlatina).

The object of this experiment was to examine the serum protein changes associated with the development of immunity. Sera from two patients in the early stages of scarlet fever were obtained and examined in the centrifuge. It was planned to obtain serum again from both patients in the later stages of the disease but owing to unforeseen circumstances this was only possible in one of the cases. This particular case (B.I.L.) was a child of four years and the first specimen of serum had a rather low total protein concentration. This fact induced us to examine the serum in the untreated state and curves were obtained which showed only slight distortion of the *A* and *X* curves. Fig. 5 *a* shows one of these curves obtained from a photograph taken 73 minutes after reaching full speed and the results are shown in Table IV.

The serum from the second case had a normal protein concentration and was therefore examined after slight dilution with 1 % sodium chloride. The results are also shown in Table IV. The sedimentation constant of the *A* fraction in the case of B.I.L.'s serum, 2·28, is again lower than the corresponding value for a normal *A* fraction obtained from Fig. 1, 2·9. Unlike the results in the earlier experiments the $S_{W,20}$ value for the *G* fraction in this case does not appear to be abnormally low. It is possible in this serum that the fraction we have labelled *X* is really an *A* fraction and that our *A* fraction is a new molecular type of lower molecular weight than serum albumin. The evidence however is inconclusive. The fractions of J.O.D.'s serum correspond satisfactorily in sedimentation constants with the fractions of normal human sera. The proportions of G fraction in both sera fall within the limits of the formula (10 ± 5) % which we have found to hold for normal sera.

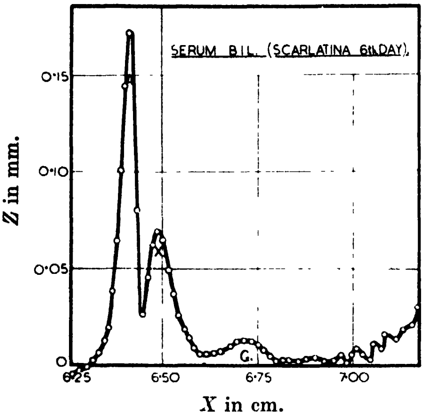

Fig. 5a.

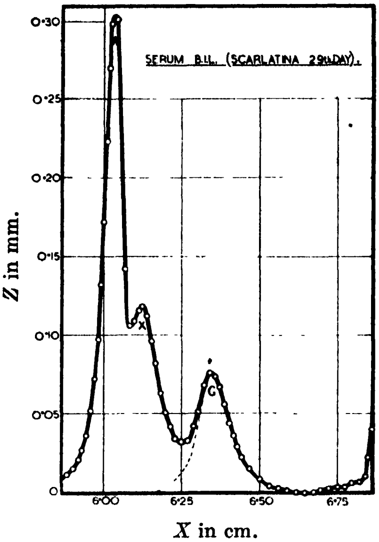

Fig. 5b.

Table IV. *Results obtained in the investigation of sera from two cases of scarlatina.*

	Serum B.I.L. (6th day of disease). Undiluted	Serum B.I.L. (29th day of disease). Diluted 5 to 2 vols. 1 % NaCl	Serum J.O.D. (8th day of disease). Diluted 5 to 2 vols. 1 % NaCl
Cell thickness (mm.)	2·00	2·00	2·00
Optical scale distance (cm.)	0·785	2·28	1·685
Cell temperature (first exposure)	33·6°	35·7°	34·2°
Cell temperature (last exposure)	36·8°	36·2°	35·6°
Approximate duration of run (hours)	3¼	3½	3
Mean centrifugal force used (times gravity)	255,000	250,000	255,000
Average $S_{W,20} \cdot 10^{13}$ A fraction	2·28	2·70	2·75
G fraction	5·08	4·95	4·90
X fraction	2·60	3·31	3·30
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\,m\mu$ A fraction	0·0070	0·0060	0·0067
G fraction	0·00102	0·0026	0·0014
X fraction	0·0046	0·0021	0·0024
Total	0·0126	0·0107	0·0105
Calculated total $n_1 - n_0$ of original serum	0·0126	0·0150	0·0147
Proportion of total $n_1 - n_0$ due to G fraction	8·1 %	24 %	13 %
Proportion of total $n_1 - n_0$ due to X fraction	36·5 %	19·5 %	23 %
Spreading coefficient of A boundary (cm.²/sec. 10⁷)	0·4	1·6	0·3

The serum of J.O.D. was dialysed at constant volume and the total protein refraction increment from refractometric measurements on serum and dialysate was found to be 0·0157 ($\lambda = 589\,m\mu$), giving 0·0169 ($\lambda = 366\,m\mu$). The percentage discrepancy between measured and calculated $n_1 - n_0$ is therefore in this experiment − 13 %.

When the undiluted serum from B.I.L. was examined 23 days later we obtained curves showing marked distortion at the A and X boundaries. These curves however showed symmetrical *G* boundary curves which gave the following value:

Calculated $n_1 - n_0$ of *G* fraction in undiluted serum (B.I.L.) ... ... 0·00336

The serum was re-examined after diluting 5 vols. of serum with 2 vols. of 1 % sodium chloride. Fig. 5 *b* shows the curve from an exposure taken 86½ minutes after reaching full speed and the general results are shown in Table IV. The distortion of the *A* and *X* boundaries has disappeared and there is obviously an increased proportion of *G* fraction present. In the period of 23 days during which immunity to scarlatina developed the total serum protein concentration has increased by 19 % of its original value.

The proportion of *G* fraction in the undiluted serum is $\frac{0{\cdot}00336 \times 100}{0{\cdot}0150} = 22{\cdot}4\ \%$.

The effect of slight dilution on the proportion of *G* fraction is not therefore very significant in this case. The large increase in the proportion of *G* fraction—from 8·1 to 22·4 %—would appear to be associated with the development of immunity to the disease.

Exp. 5. Serum from A.B. (myeloma).

This patient first came to our notice some months before the scale projection system for examining undiluted sera had been worked out. The patient's urine contained typical Bence Jones protein and we attempted an ultracentrifugal analysis of the serum in the hope of finding some evidence of the presence in it of a substance of lower molecular weight than albumin. An investigation of the urinary protein is reported in a subsequent paper. For the serum investigation we had no choice at the time but to dilute the serum, and a dilution of 2 vols. of serum with 3 vols. of Ringer's solution was found satisfactory. The curves show no evidence of the presence of an unusual molecule, but the proportion of *G* fraction is high, even for a diluted serum. The patient was lost sight of for eight months when she returned to hospital with a spontaneous fracture of the femur and obviously in the terminal stages of the disease. During this interval there had been continuous excretion of Bence Jones protein in the

Table V. *Results obtained in the examination of two specimens of serum from A.B. (myeloma)—one in the early and one in the late stages of the disease.*

		Serum A.B. Diluted 1 vol. serum to 1·5 vols. Ringer	Serum A.B. (8 months later). Diluted 5 vols. serum to 2 vols. 1 % NaCl
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		3·64	2·58
Cell temperature (first exposure)		34·5°	34·3°
Cell temperature (last exposure)		34·6°	36·3°
Approximate duration of run (hours)		2	2½
Mean centrifugal force used (times gravity)		250,000	245,000
Average $S_{\mathrm{W},20} \cdot 1{\cdot}0^{13}$	*A* fraction	3·71	2·98
	G fraction	5·44	5·11
	X fraction	—	3·38
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\, m\mu$	*A* fraction	0·0039	0·0070
	G fraction	0·0012	0·0018
	X fraction	—	0·0014
	Total	0·0051	0·0102
Calculated total $n_1 - n_0$ of original serum		0·0127	0·0143
Proportion of total $n_1 - n_0$ due to *G* fraction		23 %	18 %
Proportion of total $n_1 - n_0$ due to *X* fraction		—	14 %
Spreading coefficient of *A* boundary (cm.^{2}/sec. 10^7)		5·3	2·4

urine. We were able to obtain another specimen of serum and this was examined by the scale projection method after diluting 5 vols. of the serum with 2 vols. of 1 % sodium chloride. The diagrams again show no signs of a new molecule, the $S_{W,20}$ values for the fractions corresponding well with the values for the fractions of normal sera. The results of the two experiments are shown in Table V. The lower proportion of *G* fraction in the second experiment is probably due in part to the higher total protein concentration in which the serum was examined. In both experiments the degree of spreading at the *A* boundary appears to be unusually high. This feature will be discussed more fully later.

Exp. 6. Serum from G.L.E. (myeloma).

The results of this experiment form an interesting comparison with those of the previous one. Almost at the same time as we obtained the first specimen of serum from A.B. another clinically characteristic case of myeloma (ordinarily a comparatively rare disease) was discovered, but this case had very little protein in the urine and that present did not give the characteristic reactions of Bence Jones protein. We found the total protein concentration of the serum to be unusually high (10·9 g./100 ml.) and could only obtain satisfactory sedimentation diagrams at the time by diluting the serum with twice its volume of Ringer solution. The disease terminated fatally shortly afterwards and the *post mortem* examination confirmed the diagnosis of myeloma. The presence of Bence Jones protein in the urine however was never definitely established. We preserved a specimen of the serum in the refrigerator for some months until it was possible to undertake an examination with the scale projection system. It was then found that dilution with at least an equal volume of 1 % sodium chloride was still required to avoid completely distortion of the *A* and *X* curves.

We show in Table VI the results of the two experiments. Fig. 6 *a* shows the curve of an exposure taken 43½ minutes after reaching full speed in the experiment with the serum diluted with an equal volume of 1 % sodium chloride, and Fig. 6 *b* is from an exposure taken 26½ minutes later in the same experiment. The results show that there are present four molecular fractions in the serum, two of which correspond in sedimentation constants to the *A* and *G* fractions of normal sera. The heaviest fraction, which we have labelled *Z*, is not of much significance since it represents a very small proportion of the total protein. The remaining fraction, which we have called *Y*, is of great interest since it is clearly quite different from the *X* fraction of normal sera and of the pathological sera already described. The sedimentation constant is much higher than that of any *X* fraction in comparable circumstances which we have found and is also unusual in lying nearer to the value for the *G* fraction in the same experiment. This is also seen in Figs. 6 *a* and 6 *b* in which the separation of the *A* and *Y* peaks is greater than of the *Y* and *G* peaks and also greater than that of the *A* and *X* peaks in any of the curves previously obtained.. It is also interesting to note that the *A* curve is quite symmetrical, indicating the absence of any *X* fraction. We regard this experiment as definite evidence, to be supported by data from later experiments, that there may exist in pathological sera fractions of molecular weight not ordinarily present in normal sera.

Increasing the dilution of the serum from 1 : 1 to 1 : 2 appears to have little, if any, effect on the proportion of the *G* fraction. It appears, however, that the *Y* and *A* fractions exist in a form of concentration equilibrium just as we have shown the *G* and *A* fractions to exist in normal sera. It is not possible to examine the serum satisfactorily at a greater dilution than 1 : 2 because it will be appreciated that with three major diffusion boundaries to be accommodated in a short depth of centrifuge-cell marked overlapping must occur between them. At dilutions greater than 1 : 2 this overlapping makes graphical resolution of the component curves impossible.

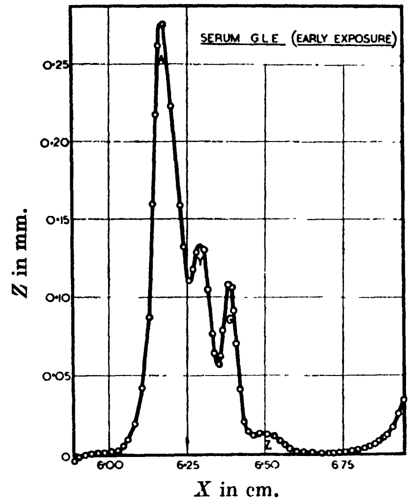

Fig. 6*a*.

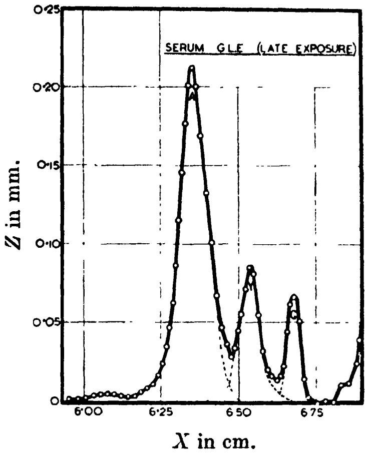

Fig. 6*b*.

Table VI. *Results obtained in the investigation of serum from G.L.E. (myeloma).*

		Serum diluted with an equal volume of 1 % NaCl	Serum diluted with 2 vols. of Ringer solution
Cell thickness (mm.)		2·00	1·50
Optical scale distance (cm.)		2·14	3·80
Cell temperature (first exposure)		33·8°	32·3°
Cell temperature (last exposure)		34·6°	34·8°
Approximate duration or run (hours)		2¼	1½
Mean centrifugal force used (times gravity)		260,000	250,000
Total $n_1 - n_0$, by refractometer $\lambda = 589\,m\mu$		0·0110	0·0073
Total $n_1 - n_0$, corrected to $\lambda = 366\,m\mu$		0·0119	0·0079
Average $S_{W,20} \cdot 10^{13}$	*A* fraction	3·09	3·64
	G fraction	5·21	6·32
	Y fraction	4·30	5·35
	Z fraction	6·43	8·46
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\,m\mu$	*A* fraction	0·00694	0·00344
	G fraction	0·00178	0·00111
	Y fraction	0·00250	0·00194
	Z fraction	0·00025	0·00024
	Total	0·01147	0·00673
"% difference" total $n_1 - n_0$		−3·4 %	−14·8 %
Proportion of total $n_1 - n_0$ due to *G* fraction		15·5 %	16·5 %
Proportion of total $n_1 - n_0$ due to *Y* fraction		21·8 %	28·8 %
Proportion of total $n_1 - n_0$ due to *Z* fraction		2·2 %	3·6 %

Exp. 7. Sera from K.M., C.P. and J.A.F. (malignant disease).

Although we have classed these three sera together under the heading of malignant disease the clinical conditions of the patients from whom they were obtained differed considerably. The first patient (K.M.) was in an extreme state of jaundice due to obstruction of the bile duct, and the serum was highly coloured with bile pigment. The second (C.P.) was emaciated and had an advanced ulcerative condition in the rectum. The third patient had a tumour in the epigastrium, but the liver was not palpable, and the general condition was not so advanced as in the other two. Further clinical details of these cases will be found in the appendix.

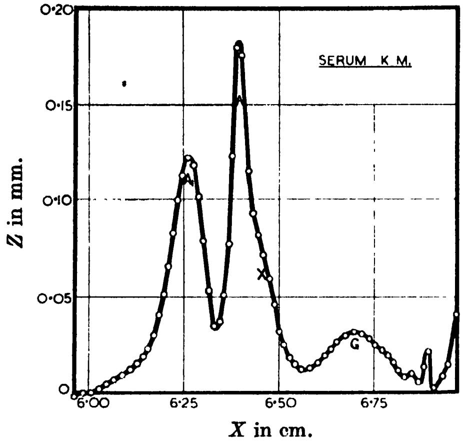

Fig. 7*a*.

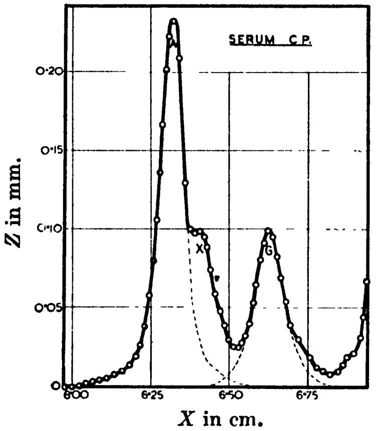

Fig. 7*b*.

Table VII. *Results obtained in the investigation of sera from three cases of malignancy.*

		Serum K.M. diluted 5 vols. serum to 2 vols. 1 % NaCl	Serum C.P. diluted 5 vols. serum to 2 vols. 1 % NaCl	Serum J.A.F. diluted 3 vols. serum to 1 vol. 1 % NaCl
Cell thickness (mm.)		2·00	2·00	2·00
Optical scale distance (cm.)		2·58	2·58	1·84
Cell temperature (first exposure)		33·2°	34·2°	33·2°
Cell temperature (last exposure)		36·3°	36·4°	34·6°
Approximate duration of run (hours)		3	$2\frac{1}{2}$	$3\frac{1}{4}$
Mean centrifugal force used (times gravity)		255,000	255,000	245,000
Total $n_1 - n_0$ of undiluted serum, by refractometer, $\lambda = 589\,m\mu$		0·0118	0·0147	0·0142
Corresponding total protein concentration (g./100 ml.)		5·84	7·28	7·03
Total $n_1 - n_0$ of diluted serum, by refractometer, and corrected to $\lambda = 366\,m\mu$		0·00910	0·0113	0·0115
Average $S_{W,20} \cdot 10^{13}$	A_1 fraction	2·57	—	—
	A fraction	3·42	2·94	2·91
	G fraction	5·58	4·96	4·99
	X fraction	3·57	3·32	3·21
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\,m\mu$	A_1 fraction	0·00290	—	—
	A fraction	0·00239	0·00525	0·00646
	G fraction	0·00127	0·00302	0·00188
	X fraction	0·00108	0·00163	0·00194
	Total	0·00764	0·00990	0·01028
"% difference" total $n_1 - n_0$		-16 %	-12·4 %	-10·4 %
Proportion of total $n_1 - n_0$ due to A_1 fraction		38 %	—	—
Proportion of total $n_1 - n_0$ due to G fraction		16·5 %	30·5 %	18 %
Proportion of total $n_1 - n_0$ due to X fraction		14 %	16·5 %	19 %
Boundary spreading coefficient of lightest fraction (cm.²/sec. × 10^7)		4·3	3·5	0·7

All three sera were diluted slightly with 1 % sodium chloride before examination in the centrifuge. The results are collected in Table VII. Fig. 7 *a* shows the curve of an exposure taken 79 minutes after reaching full speed in the first experiment (K.M.), and the curve of Fig. 7 *b* is from an exposure taken 81 minutes after reaching full speed in the second experiment (C.P.). If we consider first the results for sera C.P. and J.A.F. it will be seen that both contain three molecular fractions which correspond in sedimentation constants with the *A*, *G* and *X* fractions of normal sera. The proportion of *G* fraction in the case of J.A.F. is just above our limit for a normal serum, and in the case of C.P. there is definitely an abnormally high proportion of this fraction. The other results call for no comment.

In the serum of K.M. there are present four definite molecular types. The lightest and heaviest fractions separate well and give rise to symmetrical curves. The two intermediate fractions do not separate completely, resembling the *A* and *X* fractions of normal sera in this respect. Also, if the curve of the lightest fraction is temporarily disregarded the diagram which remains resembles very closely the diagram which we have obtained with some diluted normal sera, with the exception that the proportion of *G* fraction is greater than usual.

The $S_{W,20}$ value for the lightest fraction (which we have called A_1), 2·57, is much lower than the value for a normal *A* fraction in corresponding concentration, 3·9 (*cf.* Fig. 1). It is not possible to compare the $S_{W,20}$ value for the *A* fraction in Table VII with the value for a normal *A* fraction in corresponding concentration because the *A* protein is sedimenting in a medium of A_1 molecules. The effect of the A_1 molecules will be to reduce the sedimentation velocity of the *A* molecules. The $S_{W,20}$ value for a normal *A* fraction in a concentration equal to the sum of the *A* and A_1 fractions in this serum is 3·3 (*cf.* Fig. 1). Earlier results obtained for albumin and globulin mixtures [1935, 1] show that globulin sediments more slowly in pure solution than in a solution of the same total protein concentration but containing quantities of albumin. The value of 3·42 for the *A* fraction of this serum therefore appears to be in keeping with the view that the fraction is a normal *A* fraction sedimenting in the presence of lighter A_1 molecules. The $S_{W,20}$ values for the *X* and *G* fractions also support the view that the serum of K.M. has the qualitative constitution of a normal serum with the addition of a new molecular species sedimenting more slowly than the others. The spreading coefficient of the A_1 boundary, $4{\cdot}3 \times 10^{-7}$ cm.^{2}/sec., is consistent with the view that the fraction represents a homogeneous protein of lower molecular weight than serum albumin.

Exp. 8. Sera from G.N. and K.L. (pneumonia).

In this experiment also we are comparing the sera from patients showing marked differences in their clinical conditions. The first (G.N.) was a young man who showed all the signs of a fulminating type of pneumonia and died shortly after the serum was taken. The *post mortem* examination revealed septic infarcts in both lungs secondary to thrombo-phlebitis of the iliac vein. The other (K.L.) was from a woman of 78 years with a long history of pulmonary trouble, who was dying from croupous pneumonia with empyema.

The serum from G.N. was first dialysed at constant volume and the value of $n_1 - n_0$ obtained from refractometric measurements.

Total $n_1 - n_0$ of undiluted serum (G.N.), by refractometer ($\lambda = 589\,m\mu$) ...	0·0122
Total $n_1 - n_0$ of undiluted serum corrected to $\lambda = 366\,m\mu$	0·0132
Corresponding total protein concentration	6·04 g./100 ml.

Owing to the low concentration of total protein the serum was centrifuged without dilution. The results are shown in Table VIII and Fig. 8 shows the curve of an exposure taken 96 minutes after reaching full speed. It will be seen that three fractions are present, each of the two heavier fractions in greater concentration than the lightest one. This very severe alteration in the composition of the normal serum must have taken place inside three weeks, between the onset of the disease and the taking of the blood and was probably produced in a much shorter time.

A consideration of the $S_{W,20}$ values for the fractions shows that these are all unusually low but bear approximately the same ratio to each other as do the corresponding values for normal serum protein fractions. Once again we cannot be certain whether the fractions in this serum have abnormal molecular weights

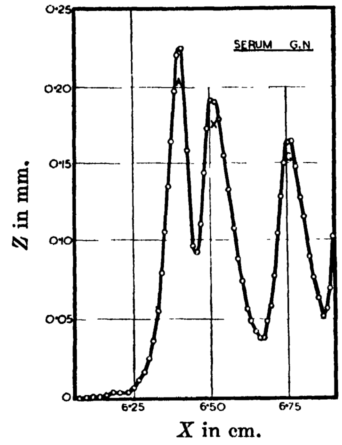

Fig. 8.

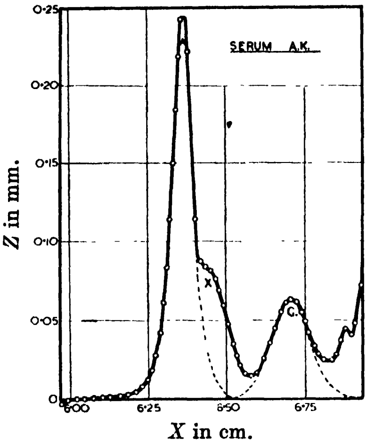

Fig. 9.

Table VIII. *Results obtained in the investigation of sera from two cases of pneumonia.*

		Serum G.N. undiluted	Serum K.L. diluted 3 vols. serum to 1 vol. 1 % NaCl	Serum K.L. diluted with an equal vol. 1 % NaCl
Cell thickness (mm.)		2·00	2·00	2·00
Optical scale distance (cm.)		3·34	1·68	3·64
Cell temperature (first exposure)		31·0°	32·7°	33·4°
Cell temperature (last exposure)		35·1°	34·4°	34·9°
Approximate duration of run (hours)		3	2¼	3
Mean centrifugal force used (times gravity)		245,000	250,000	245,000
Average $S_{W,20} \cdot 10^{13}$	*A* fraction	2·07	2·67	3·15
	G fraction	4·02	5·40	5·55
	X fraction	2·49	3·32	—
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366 m\mu$	*A* fraction	0·00361	0·00548	*0·00568
	G fraction	0·00406	0·00153	0·00112
	X fraction	0·00432	0·00272	—
	Total	0·01199	0·00973	0·00680
Calculated total $n_1 - n_0$ of original undiluted serum		—	0·0130	0·0136
Proportion of total $n_1 - n_0$ due to *G* fraction		34 %	16 %	16·5 %
Proportion of total $n_1 - n_0$ due to *X* fraction		36 %	28 %	—
Spreading coefficient of *A* boundary (cm.²/sec. × 10^7)		—	0·8	—

* Value for combined *A* and *X* fractions.

or whether a common influence is causing normal *A*, *G* and *X* fractions to sediment unusually slowly. The discrepancy ("% difference") between measured and calculated total $n_1 - n_0$ in this experiment is $-9{\cdot}1$ %.

The serum of K.L. was first examined undiluted and the curves showed distortion at the *A* and *X* boundaries. The serum was then diluted with one-third its volume and with an equal volume of 1 % sodium chloride, and the results of the two experiments are reported in Table VIII. No curves are reproduced since these show no significant points of difference from the curves of a normal serum. The results in Table VIII show that the *A* fraction of this serum sediments rather more slowly than a normal *A* fraction, while the *G* and *X* fractions correspond approximately in $S_{W,20}$ values with the *G* and *X* fractions of normal sera. It is interesting to find that the serum changes should be so small in this case of low-grade pulmonary and pleural infection, while on the other hand in the acute and rapidly fatal septic pneumonia the changes should be so radical.

Exp. 9. Sera from A.K. and E.K.L. (phthisis).

Both sera in this experiment were from elderly men with histories of pulmonary tuberculosis. A.K. was probably in a more advanced stage of the disease than E.K.L. and had been treated by induced pneumothorax. The results in Table IX show that both sera have approximately the same qualitative protein constitution as normal sera, but contain an abnormally high proportion of *G* fraction. This is especially marked in the case of A.K. A curve from this serum is shown in Fig. 9. It is from an exposure taken 85 minutes after reaching full speed. Many observers [quoted by Sato, 1933] have found a high proportion of globulin in the serum in tuberculosis using precipitation methods of analysis.

Table IX. *Results obtained in the investigation of sera from two cases of phthisis.*

		Serum A.K. diluted 5 vols. serum to 2 vols. 1 % NaCl	Serum E.K.L. diluted 3 vols. serum to 1 vol. 1 % NaCl
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		2·28	2·14
Cell temperature (first exposure)		33·2°	32·4°
Cell temperature (last exposure)		36·1°	34·3°
Approximate duration of run (hours)		2¾	2½
Mean centrifugal force used (times gravity)		255,000	250,000
Total $n_1 - n_0$ of undiluted serum, by refractometer, $\lambda = 589\, m\mu$		0·0145	—
Corresponding total protein concentration (g./100 ml.)		7·18	—
Total $n_1 - n_0$ of diluted serum, by refractometer, and corrected to $\lambda = 366\, m\mu$		0·0112	—
Average $S_{W.20} \cdot 10^{13}$	*A* fraction	2·88	2·92
	G fraction	4·98	4·96
	X fraction	3·29	3·21
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\, m\mu$	*A* fraction	0·00546	0·00619
	G fraction	0·00246	0·00164
	X fraction	0·00165	0·00112
	Total	0·00957	0·00895
"% difference" total $n_1 - n_0$		−14·5 %	—
Proportion of total $n_1 - n_0$ due to *G* fraction		26 %	18·5 %
Proportion of total $n_1 - n_0$ due to *X* fraction		17 %	12·5 %
Spreading coefficient of *A* boundary (cm.²/sec. × 10^7)		1·7	0·4

Exp. 10. Sera from P.P. and M.L. (nephritis).

Both the patients P.P. and M.L. had histories of chronic albuminuria. In the case of M.L. the undiluted serum gave distorted *A* and *X* curves. From the area of the symmetrical *G* curve we obtain the values

$n_1 - n_0$ of *G* fraction in undiluted serum (M.L.)	0·00094
Proportion of total $n_1 - n_0$ due to *G* fraction in undiluted serum	7·1 %

The sera were then examined after dilution with sodium chloride and the results are shown in Table X. No curves are reproduced since they show no unusual features. From Table X it will be seen that the $S_{W,20}$ value for the *A* fraction of P.P.'s serum, 2·98, is lower than the value for a normal *A* fraction in corresponding concentration, 3·5 (*cf.* Fig. 1). The other fractions of both sera appear to correspond in sedimentation constants to the fractions of normal sera. Serum M.L. appears to be normal in every respect. In serum P.P. the proportion of *G* fraction is higher than we have found in any normal sera. The other features are normal.

Table X. *Results obtained in the investigation of sera from two cases of nephritis.*

		Serum P.P. diluted 3 vols. serum to 1 vol. 1 % NaCl	Serum M.L. diluted 2 vols. serum to 1 vol. 1 % NaCl
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		2·58	2·44
Cell temperature (first exposure)		33·8°	32·1°
Cell temperature (last exposure)		35·3°	33·8°
Approximate duration of run (hours)		2¾	3¼
Mean centrifugal force used (times gravity)		250,000	250,000
Total $n_1 - n_0$ of undiluted serum, by refractometer, $\lambda = 589\,m\mu$		0·0122	0·0147
Corresponding total protein concentration (g./100 ml.)		6·04	7·31
Total $n_1 - n_0$ of diluted serum, by refractometer, and corrected to $\lambda = 366\,m\mu$		0·00988	0·0106
Average $S_{W,20} \cdot 10^{13}$	*A* fraction	2·98	2·83
	G fraction	5·76	5·03
	X fraction	3·77	3·14
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\,m\mu$	*A* fraction	0·00451	0·00704
	G fraction	0·00138	0·00085
	X fraction	0·00207	0·00160
	Total	0·00796	0·00949
"% difference" total $n_1 - n_0$		−20 %	−10·5 %
Proportion of total $n_1 - n_0$ due to *G* fraction		17 %	9 %
Proportion of total $n_1 - n_0$ due to *X* fraction		26 %	17 %
Spreading coefficient of *A* boundary (cm.²/sec. × 10^7)		0·3	0·6

Exp. 11. Sera from K.A.B. (congenital syphilis), H.A.A. (metastatic tumour of bone), and A.S.L. (pneumonia).

These sera are grouped together arbitrarily. The patients had no symptoms in common with exception that both H.A.A. and A.S.L. were excreting protein in the urine. The results for the serum of K.A.B. show that fractions corresponding in sedimentation constants to the *A*, *G* and *X* fractions of normal sera are present, but the proportion of *G* fraction is abnormally high. The results for the sera from H.A.A. and A.S.L. show an unusual feature. The curve in Fig. 10 *a* was obtained from an exposure in the first experiment (H.A.A.) taken 93½ minutes after reaching full speed, and that in Fig. 10 *b* is from an exposure in the second experiment (A.S.L.) taken 76½ minutes after reaching full speed. In Fig. 10 *a* the

This suggestion is confirmed by the unusually high value of the boundary spreading coefficient, $5{\cdot}03 \times 10^{-7}$ cm.^{2}/sec. We cannot resolve the combined curves graphically. For this reason the $n_1 - n_0$ value given for the A fraction in Table XI represents the value for the combined A and X fractions. A similar phenomenon appears in the serum of A.S.L. The curve (Fig. 10 *b*) is practically symmetrical and no evidence of an X peak is seen. On the other hand the general dimensions of the curve are not in keeping with a homogeneous boundary of the same protein concentration but closely resemble those for a strongly diluted human serum [*cf.* 1935, 2, Fig. 16]. The boundary spreading coefficient is again high. The impression which we have from this curve is that A and X fractions are present in the serum in concentrations which do not differ greatly from each other. We may also note the abnormally high proportion of G fraction in H.A.A.'s serum.

Exp. 12. Sera from K.J.K. (chronic nephritis) and L.E. (albuminuria of pregnancy).

The patient K.J.K. had been passing large amounts of protein in the urine for at least a year. In the patient L.E. proteinuria commenced suddenly about a month before parturition, when twins were born. During this period excessive amounts of protein were passed in the urine. Both specimens of serum were turbid in appearance and behaved in the centrifuge cell as we have described earlier in this paper for sera containing quantities of suspended lipoids. An examination of the urinary proteins will be described in a subsequent paper.

Fig. 11 *a* shows the curve of an exposure taken 65 minutes after reaching full speed in the experiment with serum, slightly diluted, from K.J.K. The complete absence of any signs of an X fraction and the marked dissymmetry of the A curve are to be noted. It occurred to us at this point that some of the unusual features of this serum might be due to the presence of quantities of urea or other waste substances in the serum. We therefore dialysed the serum (with slight incidental dilution) against changes of 1 % sodium chloride until equilibrium was obtained and re-examined the diluted serum in the centrifuge. The results of both experiments are shown in Table XII and demonstrate that no significant change has resulted from the dialysis. The general shapes of the curves for the dialysed serum are the same as those for the undialysed serum. If urea, or other dialysable substances, in serum have any effect on the serum proteins we conclude that this is irreversible.

Fig. 11 *b* shows the curve from a photograph taken 61 minutes after reaching full speed in the experiment on the serum of L.E. Once again, there are no obvious signs of an X fraction, but the general dimensions of the A curve resemble those of the boundary curve of a homogeneous protein in considerably more dilute solution. The boundary spreading coefficients in all three experiments (*cf.* Table XII) are much higher than correspond with the concentrations of the protein fractions. The large percentage discrepancies between measured and calculated total protein refraction increments also support the view that significant amounts of grossly polydisperse protein are present in both sera. It is to be noted however that the refractometric measurements of $n_1 - n_0$ include the refraction effects of the suspended lipoids which are not included in the values of $n_1 - n_0$ obtained from the centrifugal diagrams. This may introduce a significant error. The above facts, in conjunction with the dissymmetry of the A curve in Fig. 11 *a*, suggest that at least for the serum of K.J.K. the most probable explanation of the absence of any X fraction is the one suggested above—that all or a large part of the protein in the lighter fraction is polydisperse.

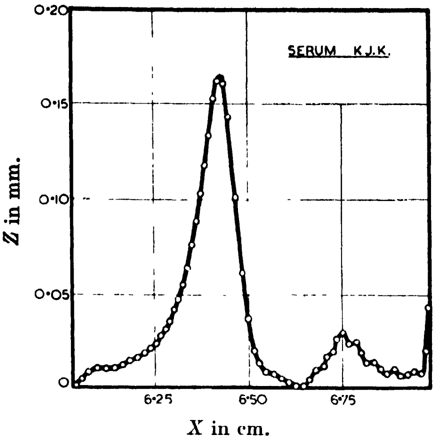

Fig. 11*a*.

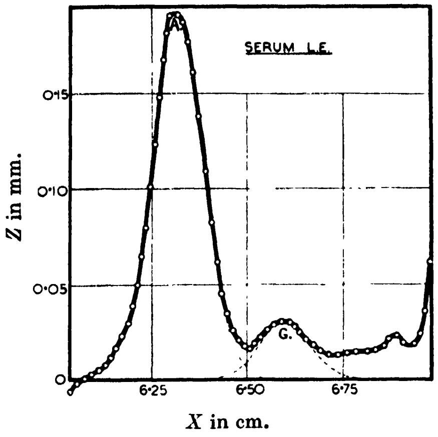

Fig. 11*b*.

Table XII. *Results obtained in the investigation of sera from K.J.K. (nephritis), and L.E. (albuminuria of pregnancy).*

	Serum K.J.K. diluted 3 vols. serum to 1 vol. 1 % NaCl	Serum K.J.K. dialysed and slightly diluted	Serum L.E. diluted 5 vols. serum to 2 vols. 1 % NaCl
Cell thickness (mm.)	2·00	2·00	2·00
Optical scale distance (cm.)	2·88	3·48	3·78
Cell temperature (first exposure)	34·6°	32·3°	33·6°
Cell temperature (last exposure)	35·7°	35·3°	35·8°
Approximate duration of run (hours)	2¼	2½	2¾
Mean centrifugal force used (times gravity)	250,000	250,000	250,000
Total $n_1 - n_0$ of undiluted serum, by refractometer, $\lambda = 589\, m\mu$	—	—	0·0126
Corresponding total protein concentration (g./100 ml.)	—	—	6·24
Total $n_1 - n_0$ of diluted serum, by refractometer, and corrected to $\lambda = 366\, m\mu$	—	0·00790	0·00972
Average $S_{W,20} \cdot 10^{13}$ *A* fraction	3·24	3·90*	3·08*
G fraction	5·61	6·65*	5·43*
Average $n_1 - n_0$, from sedimentation diagram, $\lambda = 366\, m\mu$ *A* fraction	0·00544	0·00414	0·00528
G fraction	0·00098	0·00075	0·00086
Total	0·00642	0·00489	0·00614
"% difference" total $n_1 - n_0$	—	−38 %	−37 %
Proportion of total $n_1 - n_0$ due to *G* fraction	15 %	15 %	14 %
Spreading coefficient of *A* boundary (cm.^{2}/sec. × 10^7)	6·2	10·5	8·2

* Owing to the dissymmetry of the curves these values are only approximate.

DISCUSSION.

The more important results from the foregoing experiments are collected together in Table XIII. In a comparison of these results with the results characteristic of normal human sera the following points from the data in Table XVII of the previous paper [1935, 2] are important:

Maximum proportion of *G* fraction recorded in a normal serum (total $n_1 - n_0$ of sera greater than 0·00600) = 13 %.

Maximum spreading coefficient recorded for the *A* boundary of a normal serum ($n_1 - n_0$ of *A* fractions greater than 0·00500) = $1 \cdot 9 . 10^{-7}$ cm.^{2}/sec.

Table XIII. *Collected results for pathological human sera.*

Serum	Diagnosis	Dilution	Calculated $n_1 - n_0$ of A fraction	$S_{W,20} . 10^{13}$ of A fraction	$S_{W,20} . 10^{13}$ of normal A fraction	A boundary spreading coefficient	Proportion of X fraction, %	Proportion of G fraction %
A.J.	Malignant disease	Undiluted	0·00500	2·32	3·4	—	31	26·5
B.I.L.	Scarlatina (6th day)	Undiluted	0·00700	2·28	2·9	0·4	36·5	8·1
B.I.L.	Scarlatina (29th day)	5 : 2	0·00600	2·70	3·1	1·6	19·5	24
J.O.D.	Scarlatina (8th day)	5 : 2	0·00670	2·75	3·0	0·3	23	13
A.B.	Myeloma (early)	1 : 1·5	0·00390*	3·71	3·7	5·3	—	23
A.B.	Myeloma (late)	5 : 2	0·00700	2·98	2·9	2·4	14	18
G.L.E.	Myeloma	1 : 1	0·00694	3·09	2·9	—	No X mol. 22 % new (Y) mol.	15·5
K.M.	Malignant disease	5 : 2	0·00290†	2·57†	3·9	4·3†	14 % X mol. 38 % new (A_1) mol.	16·5
C.P.	Malignant disease	5 : 2	0·00525	2·94	3·3	3·5	16·5	30·5
J.A.F.	Malignant disease	3 : 1	0·00646	2·91	3·0	0·7	19	18
G.N.	Septic pneumonia	Undiluted	0·00361	2·07	3·7	—	36	34
K.L.	Empyema	3 : 1	0·00548	2·67	3·3	0·8	28	16
A.K.	Phthisis	5 : 2	0·00516	2·88	3·3	1·7	17	26
E.K.L.	Phthisis	3 : 1	0·00619	2·92	3·1	0·4	12·5	18·5
P.P.	Nephritis	3 : 1	0·00451	2·98	3·5	0·3	26	17
M.L.	Nephritis	2 : 1	0·00704	2·83	2·9	0·6	17	9
K.A.B.	Congenital syphilis	3 : 1	0·00783	2·85	2·7	1·2	11·5	17
H.A.A.	Malignant disease	5 : 2	0·00827*	3·10	2·6	5·0	—	23·5
A.S.L.	Pneumonia	3 : 1	0·00648	3·30	3·0	3·3	0	8·5
K.J.K.	Chronic nephritis	3 : 1	0·00514	3·24	3·3	6·2	0	15
L.E.	Albuminuria of pregnancy	5 : 2	0·00528	3·08	3·3	8·2	0	14

* Combined values for A and X fractions. † Value for lightest (A_1) fraction.

There is also shown in Table XIII for each serum the value for the sedimentation constant of a normal A fraction of the same concentration as the A fraction in the serum (*cf.* Fig. 1). It will be seen that the agreement between the $S_{W,20}$ values for normal and pathological A fractions is good in some cases and very poor in others. The agreement is particularly bad in the cases of A.J., B.I.L., K.M. (A_1 fraction) and G.N. In one of these sera (K.M.) the evidence for the presence of a new molecular fraction is conclusive since four fractions are present altogether. In the other three it is possible that the fractions which we have labelled A are really of lower molecular weight than the A fractions of normal sera. It is to be noted that in these three cases also we have found abnormally high proportions of X fraction. It is possible in these cases that the fractions which we have labelled X are really A fractions and the fractions labelled A are substances of lower molecular weight. It is seen in Table XIII that the general agreement of the $S_{W,20}$ values for the lightest fractions among themselves is not as good as the agreement in the case of the A fractions of normal sera. The latter in turn do not agree to within the full experimental accuracy of the method. These facts suggest that in pathological sera, and to a lesser extent in normal sera, a variable factor of which we have no precise knowledge influences the sedimentation of the proteins. This factor does not affect the sedimentation of purified proteins in solutions of defined inorganic composition. Alternatively, there may exist in normal and pathological sera a great many A, X and G fractions of different molecular weights. To decide between these views it is necessary to carry out further investigations of the effect of the physical and chemical conditions which hold in normal and pathological sera on the sedimentation constants of the fractions. We have obtained however in the results for two sera (G.L.E. and K.M.) conclusive evidence that fractions of molecular weight not ordinarily found in normal sera may be present in pathological sera.

When we consider the concentration relationships of the fractions in pathological sera it is seen in Table XIII that with the exception of the early scarlatinal sera and those from M.L. (nephritis) and A.S.L. (pneumonia) all the sera have higher proportions of G fraction than normal. On the one hand this is not surprising in view of the severity of most of the conditions; on the other, it is interesting to find these increased proportions of G fractions in diseases such as malignancy to which immunity is not ordinarily developed. It does not seem possible to associate the increased proportions of the G fractions with a purely protective mechanism. The increases occur in a wide variety of conditions. There seems to be nothing specific therefore about the occurrence of a high proportion of G fraction in pathological sera. Various observers have come to the same conclusion with regard to the proportion of globulin in pathological sera as estimated by precipitation with neutral salts (see reviews by Peters and Van Slyke [1931] and Boyland [1932].

In the various experiments of this series we have recorded a wide variety of values for the proportions of the X fractions, many of them lower and many higher than the extreme values obtained in our series of normal sera. We hesitate however to base any important conclusions on our individual figures because in many cases an element of uncertainty attaches to the process of graphical separation of the X curves from overlapping A curves. The general deduction is drawn from the values obtained that there exist in pathological sera variations in the proportions of X fractions which are wider than the variations found in normal sera.

A voluminous literature has been compiled in recent years by various observers [Schade and Clausen, 1924; Govaerts, 1925; Farkas, 1927; Krogh and Nagazawa, 1931; Kylin and von Pein, 1931; Iversen, 1932; to mention only a few contributions] on the colloid osmotic pressure of normal and pathological sera. A few of these observers have calculated the osmotic pressure per gram of serum protein and have deduced from this figure the presence of an abnormal concentration of the albumin fraction or of the globulin fraction as the case may be. It cannot be said that the application of this method has yielded consistent results and the centrifugal data furnish an explanation of this. So long as there are more than two variables, *i.e.* more than two molecular fractions in the serum, a measurement of the osmotic pressure (presuming this to be reliably corrected for the effects of the diffusible ions) can give no idea of the relative concentrations of the fractions. The method cannot be applied to normal sera because of the presence of A, G and X fractions, and may be applied still less to pathological sera in which additional fractions may be present. It appears to us that the recorded measurements of colloid osmotic (oncotic) pressure in pathological sera can have little precise significance in relation to the protein composition of the sera.

Consideration of the spreading coefficients in Table XIII shows that the following sera, all with A fraction n_1-n_0 values greater than 0·00500, have abnormally high A boundary spreading coefficients; A.B. (myeloma), C.P. (malignancy), H.A.A. (malignancy), A.S.L. (pneumonia), K.J.K. (nephritis) and L.E. (albuminuria of pregnancy). The variety of the pathological conditions indicates that this physico-chemical index appears to have no specific disease relationship. The striking fact however is that with the exception of C.P., of whom unfortunately we have no accurate urinary details, all the patients are passing protein in the urine. It appears probable that the poly- or hetero-disperse protein of the A fractions in these sera is capable of passing through the kidneys. On the other hand we cannot say that poly- or hetero-dispersity of serum protein is an essential condition for proteinuria because in our series there are two cases of chronic albuminuria (P.P. and M.L.) in which the spreading coefficients of the

A boundaries are normal. We give some further important evidence on this subject in a later paper dealing with the analysis of urinary proteins.

We believe that the existing methods of serum protein analysis by precipitation with neutral electrolytes do not give rise to fractions of even approximately uniform composition and therefore the results have a doubtful significance. Our evidence for this will be given in a later paper. The question arises whether the results of ultracentrifugal analysis are likely to have a greater significance. In the first place we may expect the results to be consistent since they represent measurements of a single well-defined property of serum, *viz.* the molecular dimensional distribution of the protein. Secondly, they have the merit of being obtained in circumstances which involve little, if any, change in the physico-chemical properties of the environment of the protein. This helps to eliminate arbitrary assumptions. It is true that the chemical natures of the fractions of concentrated sera which separate in the centrifugal field are not determined. This constitutes an important limitation in the interpretation of the results which we hope will be eliminated in the future. On the other hand, it is conceivable that the concentrations of molecules of a few particular sizes—no matter of what chemical constitution—may be of great significance. When one considers the relationship of protein molecular size to the viscosity and colloid osmotic pressure of serum, and the vital functions which those two serum properties have in the organism, it is realised that the results of the purely molecular dimensional analysis of serum protein may be of considerable importance. Finally, it is clear that in the consistent measurement of a single property of serum—no matter if the true significance of the property is obscure—there is a higher probability of finding an empirical relationship with some general or local condition in the organism, and this is frequently the object of serum protein analysis. For these reasons we believe that the ultracentrifugal method of analysis is at present a more rational procedure to use in the investigation of pathological sera than the precipitation method of analysis.

We have shown that the concentration values of the protein fractions obtained in the ultracentrifuge are dependent on the protein concentration in which the serum is examined. This dependence of the results on protein concentration means that it is necessary to use one of three procedures in order to give the results for various sera a comparative significance. We must determine the molecular dimensional distribution of the sera, by direct or indirect methods: (1) in the undiluted state; (2) in a state of dilution such that no change in the concentration of the fractions occurs on further dilution; or (3) at a constant concentration of protein. The second procedure is a difficult one in practice as the graphical analysis of the serum diagrams is often unsatisfactory, or impossible, in the case of highly diluted sera. Frequently also, there occur irregularities in the diagrams which are of doubtful significance. The third is not always ideally applicable because it is impossible by mere dilution of two sera each containing several fractions to obtain comparable conditions for all the fractions. The method is nevertheless frequently capable of giving valuable comparative information, and it is proposed in the continuation of these studies in the future to depend on a combination of this method and of the first procedure mentioned above.

SUMMARY.

A series of pathological sera from cases representing a variety of diseases has been examined in the ultracentrifuge.

1. In the cases of two antidiphtheritic horse sera examined without treatment of any kind it was found that the protein is approximately equally distri-

buted between an albumin and a globulin fraction. Dilution of the sera causes a marked increase in the proportion of the globulin fraction.

2. The majority of the pathological human sera examined contain three molecular fractions. In some of these sera the fractions have the sedimentation constants characteristic of the *A*, *G* and *X* fractions of normal human sera. In others the sedimentation constants are different indicating either that the fractions have different molecular weights or that some unknown and variable factor is present in pathological serum which affects the sedimentation rates of the fractions.

3. In two sera definite evidence has been found of the presence of fractions of molecular weight not normally found in human sera. In one case of malignant disease 30 % of a substance of substantially lower molecular weight than serum albumin was present in addition to the *A*, *G*, and *X* fractions. In another case of myeloma 22 % of a substance of molecular weight intermediate between the *X* and *G* fractions of normal sera was present. In this case *A* and *G* fractions were present but no *X* fraction.

4. In a considerable majority of these pathological sera the proportions of the total proteins in the untreated or slightly diluted sera represented by the *G* fractions are greater than in normal sera. The increase in the proportion of *G* fraction appears to have no special relationship to one or more diseases but to represent a general form of reaction to a number of unrelated pathological states.

5. A wide variety of values for the proportions of *X* fractions are recorded and it is evident that in a number of diseases the proportion of this fraction does not fall within the limits for a normal serum.

6. Some of the sera examined react more readily to simple dilution than do normal sera. The nature of this reaction consists as in normal sera in an increase in the proportion of *G* fraction at the expense of the lighter fractions. Fractions not normally present in serum appear to exist also in equilibrium with the other fractions.

7. Untreated pleural exudate from a case of malignancy behaved in the centrifuge like the serum of the same patient diluted to the same concentration of total protein.

8. Certain sera show an unusual phenomenon which takes the form of an abnormal spreading of the overlapping *A* and *X* boundaries. There is evidence that this is partly or wholly due to the presence of polydisperse protein molecules of mean molecular weight approximately the same as that of the *A* fraction. In four out of five such cases protein was present in the urine.

APPENDIX OF CLINICAL NOTES.

A.J. Female. 60 years. Ascites since December, 1933, with loss of weight. 21. ii. 34: large tumour palpable on right side of abdomen, exudate in left pleura, no signs of pulmonary metastases on X-ray examination, trace of protein in urine but this was not consistently present. 17. iv. 34: many large abdominal tumours palpable suggesting peritoneal metastases. Died 29. iv. 34. Permission for P.M. refused. Diagnosis: ovarian tumour with secondary metastases. Blood taken for centrifugal examination of serum on 18. iii. 34.

K.H.B. Male. 23 years. Very tired in summer of 1933 and unfit for work, later became hoarse. December, 1933: blood in sputum and attacks of shivering. 19. ii. 34: temperature 104° F., pulse 100–120, sputum T.B. + +, X-ray showed advanced T.B. in both lungs. Died 27. iii. 34. P.M.: cavities in both lungs, tuberculous ulceration of larynx, trachea and intestines, inflammation of spleen. Blood taken on 16. iii. 34.

B.I.L. Male. 4 years. 16. v. 34: headaches and throat pains. 17. v. 34: rash on chest and back, temperature 103° F., pulse 120. Diagnosis: typical scarlatina. Blood taken on 22. v. 34 and again on 14. vi. 34.

J.O.D. Male. 11 years. 10. v. 34: throat pains, difficulty in swallowing. 11. v. 34: typical rash all over body, swollen submaxillary glands, inflamed throat and tonsils. 18. v. 34: desquamation, temperature 100–102° F., pulse 90–120. Diagnosis: typical scarlatina. Blood taken on 18. v. 34.

A.B. Female. 47 years. Pains in lumbar region and chest for one year until 10. xi. 33. Urine gave typical Bence Jones precipitate at 50°, clear at 100°, 6–12 $^{\circ}/_{oo}$ Esbach, non-protein nitrogen 35 mg./100 ml., B.P. 170/140. X-ray on 20. xi. 33 showed enlargements on 1st and 2nd ribs on right side and several tumours of pelvic bones accompanied by much destruction. 17. iii. 34: spontaneous fracture of right femur, X-ray showed neck and trochanter destroyed. Patient still alive on 27. vi. 34. Diagnosis: myeloma. Blood taken on 18. xi. 33 and on 27. vi. 34.

G.L.E. Male. 68 years. January, 1933: pain in lumbar region and both shoulders, girdle pain at 11th and 12th thoracic vertebrae, later also at 8th and 9th thoracic and 2nd and 3rd lumbar, poor appetite but no emaciation, dyspnoea, coughing and hoarseness but no X-ray evidence of pulmonary metastases, liver slightly enlarged, prostate normal, symmetrical atrophy of both legs, marked osteoporosis of vertebrae with compression of several in lumbar and upper thoracic regions, several ribs quite soft, fracture of 8th and 9th, old fracture of 4th (right side), only a trace of protein in the urine (giving slight opalescence at 67°, definite at 70°, and no change at 100°), Esbach 0·5 $^{\circ}/_{oo}$. Died 8. x. 33. P.M.: medullary tumour (myeloma). Blood taken on 9. ix. 33.

K.M. Male. 78 years. Increasing tiredness and emaciation for a month. 13. v. 34: intense jaundice, urine dark brown and frothy, clay-coloured stools, plasma colour equivalent to 71 Meulengracht, mobile tumour in epigastrium (finger-thick, cord-shaped, and very hard). Diagnosis: malignant tumour of bile duct. Blood taken on 15. v. 34.

C.P. Female. 67 years. October, 1933: pains at stool, constipation and diarrhoea alternately, mucus and blood in faeces. 15. v. 34: tired, pale and emaciated, 5 inches inside rectum hard stenotic tumour with finger-wide central opening and ulcerated. Diagnosis: rectal carcinoma. Blood taken on 15. v. 34.

J.A.F. Male. 62 years. 10. xii. 33: complained of weakness. 19. iii. 34: emaciated and pale, blood in faeces, palpable tumour in epigastrium, liver not palpable, temperature 100° F., treated with cobra venom. Diagnosis: gastric carcinoma. Blood taken on 6. iv. 34.

G.N. Male. 28 years. 20. ii. 34: temperature 100–104° F., pulse 110–140, a trace of protein in urine. 6. iii. 34: two pleural punctures without result, B.P. 115/70. Died 19. iii. 34. P.M.: thrombophlebitis of iliac vein, bilateral emboli of pulmonary artery and large infarcts, organised pneumonia on right side and acute on left secondary to infection of infarct, acute nephritis. Blood taken on 13. iii. 34.

K.L. Female. 78 years. 10. iii. 34: rigors, temperature 102° F., 12. iii. 34: 270 ml. purulent fluid obtained from right pleural cavity. Died 20. iii. 34. P.M.: cardio-arterio-sclerosis, croupous pneumonia, mucopurulent bronchitis, emphysema, pleural empyema, uterine fibromyomata, ovarian cyst. Blood taken on 18. iii. 34.

A.K. Male. 59 years. Dry pleurisy in 1930. Feb. 1934: much coughing, phthisis diagnosed, sputum T.B. + + +, patient treated with pneumo-thorax. 27. vi. 34: still in hospital. Blood taken on 11. v. 34.

E.K.L. Male. 69 years. In sanatorium in April 1922. 14. iii. 34: temperature 97–98° F. in morning, 100–101° F. in evening, pulse 72–120, X-ray showed T.B. in upper third of both lungs with large cavities, sputum T.B. +. Discharged rather better 1. v. 34. Diagnosis: chronic pulmonary tuberculosis. Blood taken on 21. iv. 34.

P.P. Male. 65 years. Dyspnoea for 10 years with intermittent oedema and palpitations. 11. iv. 34: oedema, ascites, enlargement of liver, pleural effusion, Esbach 3–7 $^{\circ}/_{oo}$, non-protein nitrogen 120 mg./100 ml., W.R. negative. Went home rather better on 12. v. 34. Diagnosis: chronic glomerulo-nephritis. Blood taken 20. iv. 34.

M.L. Female. 45 years. Protein in urine and high blood pressure since 1931. 16. iii. 34: slight oedema of legs, no eye symptoms, Esbach $1\,^{\circ}/_{oo}$, blood and casts in urine, non-protein nitrogen 37·5 mg./100 ml., B.P. 165–100. Went home rather better on 3. v. 34. Diagnosis: recurrent nephritis (? acute). Blood taken on 18. iii. 34.

K.A.B. Male. 17 years. Partially deaf in both ears, becoming quite deaf in right ear in 1927. 1933: accident to knee, swollen and painless, yellow fluid in joint (Esbach $2{\cdot}5\,^{\circ}/_{oo}$), C.S.F. gave negative protein reactions, W.R. +, Kahn + +. Treated with malaria on 24. iii. 34. Diagnosis: congenital syphilis. Blood taken on 23. iii. 34.

H.A.A. Male. 59 years. Pain in right leg in January, 1934, with later pain in lumbar region. 6. iii. 34: movements impaired, emaciated, X-ray showed collapse of vertebrae with atrophy and sclerosis of surrounding tissue. No protein in urine until 3. v. 34 and thereafter only present intermittently in small amounts. Patient was treated with snake venom but died on 1. vi. 34. P.M.: sympathico-gonioma in region of superior ganglion with metastases in ribs and vertebrae, cardio-arterio-sclerosis. Blood taken on 9. v. 34.

A.S.L. Male. 27 years. Treated five times for croupous pneumonia. 1. iii. 34: acute attack of shivering and pains in chest, haemorrhagic sputum, X-ray showed pneumonia in upper lobe of right lung, typical pneumonia temperature chart, temperature normal on 8. iii. 34, urinary protein up to $16\,^{\circ}/_{oo}$ Esbach, non-protein nitrogen 33–40 mg./100 ml. Went home better on 11. v. 34. Diagnosis: pneumonia. Blood taken on 26. iv. 34.

K.J.K. Male. 42 years. October, 1933: oedema of legs, eye symptoms, swollen abdomen, tiredness, dyspnoea. 16. i. 34: oedema of penis, scrotum, and legs, urine 600–1700 ml. per day, protein always present up to $3\text{–}15\,^{\circ}/_{oo}$ Esbach, casts and a few red cells, B.P. 145/90–180/110. Diagnosis: chronic nephritis. Blood taken on 24. iv. 34.

L.E. Primipara. 21 years. No miscarriages. Protein first found in urine on 20. iv. 34, thereafter present every day in large amounts (Esbach $10\text{–}12\,^{\circ}/_{oo}$), no eclampsia, B.P. 165/90. Twins were born on 16. v. 34. Diagnosis: albuminuria of pregnancy. Blood taken on 12. v. 34.

REFERENCES.

Boyland (1932). *Cancer Rev.* **433.**

Farkas (1927). *Z. exp. Med.* **53**, 666.

Govaerts (1925). *Compt. Rend. Soc. Biol.* (*Soc. Belge Biol.*), **93**, 441.

Iversen (1932). *Acta Med. Scand.* **78**, 297.

Krogh and Nagazawa (1931). *Biochem. Z.* **188**, 241.

Kylin and von Pein (1931). *Z. exp. Med.* **76**, 549.

McFarlane (1935, 1). *Biochem. J.* **29**, 407.

—— (1935, 2). *Biochem. J.* **29**, 660.

Peters and Van Slyke (1931). Clinical methods, **2.** (Ballière, Tindal and Cox.)

Sato (1933). *Tohoku J. Exp. Med.* **22**, 116.

Schade and Clausen (1924). *Z. klin. Med.* 5, 363.

CXLVII. THE BEHAVIOUR OF URINARY PROTEINS IN THE ULTRACENTRIFUGE.

By ARTHUR SPROUL McFARLANE[1].

From the Institute of Physical Chemistry, Upsala, Sweden.

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The question whether the proteins found in the urine in certain diseases have the same composition as the proteins in the blood is one of great importance. With the probable exception of two conditions, *viz.* orthostatic and Bence Jones proteinuria, it is believed that the presence of protein in the urine may be taken as a sign of impaired renal function. If the urinary protein is the same as the plasma protein it is reasonable to assume that the disease has damaged the kidneys in such a way as to give rise to a form of leakage of plasma into the urinary tract. If the protein is different, leakage of a gross type can be excluded and it is not at all clear how the protein finds its way into the urine. We may assume that the composition of the plasma protein is changed in the renal cells by the disease process at the same time as the protein is excreted; or we may postulate that the altered urinary proteins arise in remote parts of the circulation and are excreted by normal kidneys. Since the presence of Bence Jones protein in the blood has been demonstrated in certain cases, the group of diseases with which this protein is characteristically associated has been regarded as unique (if we except the orthostatic albuminurias) in conforming to the latter hypothesis, although a few observers [Forbes *et al.*, 1933] identify renal changes with Bence Jones proteinuria. Kerridge and Bayliss [1932] have shown that Bence Jones protein readily passes through the kidneys of the cat while normal human serum proteins do not. Kerridge [1934] has shown also that the urinary proteins from ten cases of chronic nephritis did not pass through the kidneys of the cat. The inference from this (which incidentally Kerridge avoids making) is that the proteins in the urine in chronic nephritis are the same as the proteins in the plasma, and that the theory of leakage of plasma protein through the kidneys holds. The view of the identity of urinary and plasma proteins is supported by many observers. Hewitt [1927; 1929] has shown that urinary and serum albumins from cases of chronic nephritis have the same optical rotatory powers. Smith, quoted by Hewitt [1929], has shown that they have the same absorption spectra. Cavett and Gibson [1931] have shown that they have similar racemisation curves. Widdowson [1933] has investigated the optical rotation, racemisation, osmotic pressure and specific refraction of urinary and serum protein fractions in nephritis and nephrosis and concludes that the proteins from urine and serum are identical. Kozawa *et al.* [1933] claim to have shown that the amino-acid composition, albumin/globulin ratio and isoelectric(?) point of urinary proteins are the same as those of serum proteins.

Nevertheless, there are certain points which are not explained. The fibrinogen of plasma is never found in the urine. The urinary proteins seldom have the same albumin/globulin ratio as the serum proteins, and not infrequently globulin is absent altogether from the urine. It thus becomes necessary to postulate a selective permeability of the leaking kidneys to the different protein fractions of serum. Kerridge and Bayliss [1932] have put forward evidence in support of the view that the renal glomeruli function as graded filters capable of allowing molecules smaller than serum albumin to pass and withholding all others. This theory fails to explain the ready passage of haemoglobin (which has approximately the same molecular weight as serum albumin) through the kidneys, and we shall show in due course that molecules of greater dimensions than serum albumin are undoubtedly found in the urine. A major criticism of theories based on the dimensions of protein molecules is that one can never be sure of the physico-chemical conditions in the renal cells across which the proteins must pass from blood stream to urine. The proteins as a class are rather prone to molecular dimensional changes in unusual environmental circumstances, particularly when the hydrogen ion concentration is abnormal.

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[1] Beit Memorial Research Fellow.

We have described in a previous paper [McFarlane, 1935, 3] an unusual phenomenon which appeared when serum protein from certain patients passing protein in the urine was examined in the ultracentrifuge. This consisted in an abnormal degree of spreading of the *A* boundary which suggests that the "albumin" fraction is wholly or in part heterogeneous in respect of the dimensions of the constituent molecules. A less likely possibility that the molecules of the fraction are homogeneous but are endowed with an abnormal power of diffusion cannot, however, be excluded. Whatever may be the interpretation of the phenomenon the appearance denotes a fundamental change in the serum protein and it is clearly of interest to find if any similar appearance can be detected in the urinary protein. We shall report in this paper the ultracentrifugal analysis of five specimens of urinary protein obtained from patients whose sera had all been examined and reported on in the previous paper [1935, 3]. The clinical notes relating to these patients will be found appended to that paper.

The general procedure adopted was to dialyse a sample of the fresh urine in a collodion sac against 1 % sodium chloride and under pressure until equilibrium between protein solution and dialysate was reached. The refractive indices of solution and dialysate were then measured, and the solution was examined in the ultracentrifuge. The subsequent procedure was exactly as detailed in previous papers [1935, 1, 2, 3] for serum proteins.

Approximate values for the concentrations of albumin and globulin fractions in the urinary proteins were obtained by the following procedure. The dialysed and concentrated urinary protein solution was diluted with ten times its volume of 1 % sodium chloride and an equal volume of saturated ammonium sulphate added. Precipitation was given 2–4 hours for completion and then the mixture was centrifuged until the globulin precipitate was well packed down on the bottom of the tube. The supernatant albumin solution was poured off and the precipitate drained carefully and redissolved in water. The globulin solution was then dialysed against 1 % sodium chloride and the concentration of globulin measured refractometrically. Only in the cases of two of the urines (K.J.K. and L.E.) was a significant globulin precipitate obtained. The other urines appeared to contain only albumin.

Exp. 1. Urinary protein from A.S.L. (pneumonia and nephrosis) and A.B. (myeloma).

Neither urine in this experiment gave a globulin precipitate but the urinary protein of A.B. had all the characteristics of Bence Jones protein, precipitating at 50° and redissolving completely at 100°. We isolated the protein from this urine by the following procedure. To 1 volume of urine 2 volumes of saturated ammonium sulphate were added, and the precipitate was centrifuged off after

4 hours and washed with 66 % saturated ammonium sulphate. The precipitate was redissolved in a small volume of water, the turbid solution filtered and the protein reprecipitated with a large excess of 66 % saturated ammonium sulphate. The precipitate was redissolved and second and third reprecipitations carried out in the same manner. The final solution was dialysed until free of sulphate ions and electrodialysed for 4 hours at 440 volts. The liquid was pale brown and clear. Before examining a portion of this in the ultracentrifuge, sufficient solid sodium chloride was added to it to make a 0·9 % solution.

Table I shows the collected results for experiments on the two specimens of dialysed whole urinary protein and on the preparation of Bence Jones protein. Fig. 1 *a* shows the curve of an exposure taken $54\frac{1}{2}$ minutes after reaching full speed in the experiment on the urinary protein of A.S.L. The corresponding curve in the case of A.B. shows no significant points of difference from this curve. Fig. 1 *b* shows the curve of an exposure taken 81 minutes after reaching full speed in the experiment on Bence Jones protein. The curves of these proteins show no signs of a globulin fraction, thus differing markedly from the curves of the parent sera [*cf.* 1935, 3, Fig. 9 *b*]. They also show no signs of the presence of an

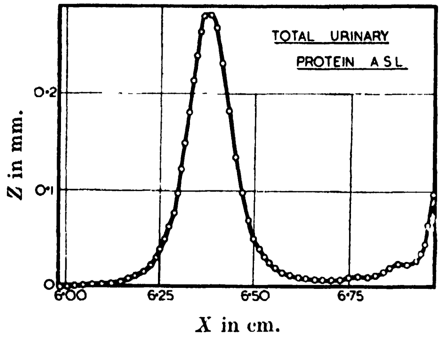

Fig. 1*a*.

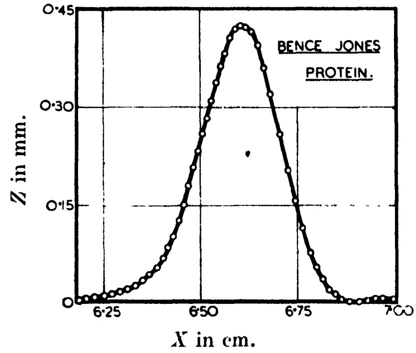

Fig. 1*b*.

Table I. *Results obtained in the investigation of urinary proteins from A.S.L. (pneumonia and nephrosis), and A.B. (myeloma).*

	Concentrated urinary protein from A.S.L.	Concentrated urinary protein from A.B.	Bence Jones protein A.B.
Cell thickness (mm.)	2·00	2·00	6·00
Optical scale distance (cm.)	3·18	2·58	7·64
Cell temperature (first exposure)	33·5°	35·5°	26°
Cell temperature (last exposure)	35·3°	40·4°	29°
Approximate duration of run (hours)	$2\frac{1}{4}$	$3\frac{1}{2}$	$2\frac{1}{2}$
Mean centrifugal force used (times gravity)	250,000	240,000	210,000
Total $n_1 - n_0$ of concentrated urine, by refractometer $\lambda = 589\,m\mu$	0·00812	0·0114	—
Corresponding total protein concentration (g./100 ml.)	4·02	5·64	—
Total $n_1 - n_0$ corrected to $\lambda = 366\,m\mu$	0·00877	0·0123	—
Average $S_{W,20} \cdot 10^{13}$ of protein	3·34	3·26	3·74
Average $n_1 - n_0$ of protein calculated from area of curve ($\lambda = 366\,m\mu$)	0·00788	0·0116	0·00295
"% difference" total $n_1 - n_0$	−10 %	−5·7 %	—
Spreading coefficient of A boundary (cm.²/sec. × 10⁷)	3·5	9·3	22·6

X protein, which is a point of similarity with the parent serum in the case of A.S.L. The sedimentation constants of the urinary proteins appear to be slightly higher than corresponds to the *A* fractions in the parent sera. The most important point, however, is the high values of the boundary spreading coefficients. These are much higher than correspond to homogeneous boundaries of the same sedimentation constants. Figs. 1 *a* and 1 *b* show the high ratio of breadth at the base to height in these curves. The urinary proteins in these cases therefore show essentially the same phenomenon as the *A* fractions in the parent sera. In the case of A.S.L. the spreading coefficient of the urinary protein boundary is in good agreement with the value for the *A* boundary of the serum. This is not so with the serum and urine of A.B. It must be realised, however, that the serum *A* fraction may contain both homogeneous and polydisperse protein, whereas the urinary protein may only contain the latter and would in that case have a larger boundary spreading coefficient.

The boundary spreading coefficient in the case of the purified Bence Jones protein is very high. The total protein concentration however is low, and the experiment was carried out with a lower mean centrifugal force than usual. The value may not therefore be compared with the others, but it is higher than that of horse serum albumin in the same circumstances. The $S_{W,20}$ value corresponds with the value for horse serum albumin in the same concentration. It is definitely higher than the values obtained by Svedberg and Sjögren [1929] in the examination of a specimen of Bence Jones protein which had the same mean molecular weight as egg albumin, *viz.* 35,000. It is also interesting to find that the isoelectric point of this specimen of Bence Jones protein as measured by Tiselius is at p_H 5·20, whereas the isoelectric point of our protein, measured by Pedersen, is at p_H 6·56. It appears that the term Bence Jones protein does not always refer to the same substance, but that urinary protein of varying composition can give the characteristic Bence Jones reactions. Some specimens of Bence Jones protein for instance crystallise easily while others cannot be crystallised. Svedberg and Eriksson-Quensel (private communication) have had a sample of Bence Jones protein which proved to be quite polydisperse. Mainzer [1932, 1, 2, 3], by investigating the electrometric titration values and transport cataphoresis of Bence Jones protein, also comes to the conclusion that it is not a chemical individual.

Exp. 2. Urinary protein from K.J.K. (chronic nephritis), L.E. (albuminuria of pregnancy), and H.A.A. (metastatic tumour of bone).

In the precipitational analysis of two of these sera (K.J.K. and L.E.) substantial globulin precipitates were obtained while the urinary protein of H.A.A. gave only a faint opalescence. The results of the analysis were

Proportion of globulin in urinary protein of K.J.K., 19 %
Proportion of globulin in urinary protein of L.E., 14 %
Proportion of globulin in urinary protein of H.A.A., less than 2 %

The results obtained in the investigation of one specimen of dialysed and concentrated whole urinary protein from each of the three patients are shown in Table II.

Fig. 2 *a* shows the curve of an exposure taken 115½ minutes after reaching full speed in the experiment on K.J.K.'s urinary protein, and Fig. 2 *b* shows the curve of an exposure taken 149 minutes after reaching full speed in the experiment on H.A.A.'s urinary protein. The curves in the third experiment (L.E.) are very similar to those for K.J.K., and differ significantly from the curves in

Figs. 1 *a* and 1 *b* in showing the presence of a *G* fraction. No significance attaches to the fact that the proportion of *G* fraction in the urines is not the same as in the original sera, because it is probable that in the precipitation method of analysis a globulin fraction is obtained which is contaminated with considerable amounts of albumin.

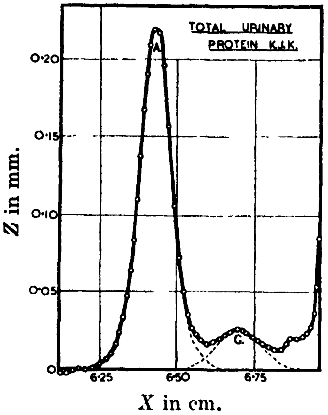

Fig. 2*a*.

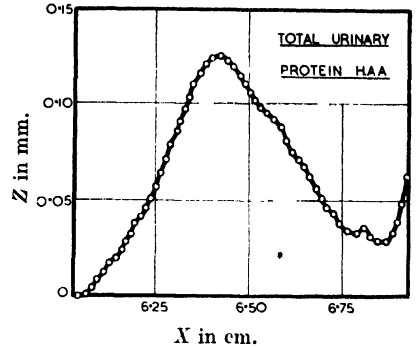

Fig. 2*b*.

Table II. *Results obtained in the investigation of urinary proteins from K.J.K. (nephritis), L.E. (albuminuria of pregnancy), and H.A.A (metastatic tumour of bone).*

		Concentrated urinary protein from K.J.K.	Concentrated urinary protein from L.E.	Concentrated urinary protein from H.A.A.
Cell thickness (mm.)		2·00	2·00	2·00
Optical scale distance (cm.)		1·68	1·98	6·78
Cell temperature (first exposure)		32·9°	33·3°	33·0°
Cell temperature (last exposure)		36·2°	36·9°	37·3°
Approximate duration of run (hours)		3¼	2½	3
Mean centrifugal force used (times gravity)		255,000	255,000	255,000
Total $n_1 - n_0$ of calculated urinary protein, by refractometer, $\lambda = 589 m\mu$		0·0107	0·0110	0·00780
Corresponding total protein concentration (g./100 ml.)		5·30	5·45	3·86
Total $n_1 - n_0$ corrected to $\lambda = 366 m\mu$		0·0115	0·0119	0·00842
Average $S_{W,20} \cdot 10^{13}$	*A* fraction	3·02	2·95	1·44
	G fraction	5·41	4·77	—
Average $n_1 - n_0$, calculated from sedimentation, diagram $\lambda = 366 m\mu$	*A* fraction	0·00971	0·00904	0·00567
	G fraction	0·00150	0·00066	—
	Total	0·01121	0·00970	0·00567
"% difference" total $n_1 - n_0$		-2·6 %	-18·5 %	-33 %
Proportion of total calculated $n_1 - n_0$ due to *G* fraction		13 %	6·8 %	—
Spreading coefficient of *A* boundary (cm.²/sec. × 10⁷)		2·7	4·9	18·5

It is obvious that the urinary protein of H.A.A. is almost completely polydisperse. Considerable amounts of protein had reached the bottom of the cell before all the protein had left the region of the meniscus. The $S_{W,20}$ value has little meaning in such a case because of the asymmetry of the curve and the doubtful significance of the apex at any instant. The value recorded in Table II, *viz.* 1·44, is obtained from measurements of the rate of movement of the apex.

Also, the apparent area of the curve is not constant but diminishes steadily as the experiment progresses. The values for $n_1 - n_0$ are all much lower than the measured total $n_1 - n_0$ value of the original preparation. The $n_1 - n_0'$ value in Table II corresponds to the mean area. The boundary spreading coefficient is calculated from the mean area and from the height of the apex above the base line in two exposures.

The *A* and *G* fractions in the other two urines have sedimentation constants corresponding approximately to those of the *A* and *G* fractions in the sera from the same patients. The *A* boundary spreading coefficients are lower in the urinary proteins but this is not inconsistent with the much higher concentrations in which they were examined. The values are greater than those for horse serum albumin in corresponding concentrations, and a comparison of the general dimensions of the curves with those of *A* curves of normal human serum of the same, or slightly smaller, total protein concentration [*e.g.* 1935, 2, Fig. 15] confirms that abnormal spreading of the *A* boundaries is present.

DISCUSSION.

We conclude from the results of these experiments that in the cases examined there is a difference between urinary protein and normal serum protein. The difference consists in an abnormal degree of spreading in the centrifugal field of the lighter fraction in the urinary protein. There is evidence that the same abnormal spreading characterises the *A* fractions in the sera of the patients. But the total urinary and serum proteins from the same patients have not identical compositions. In three cases no globulin fraction is present in the urine although in two of the three there is an abnormally high concentration of globulin in the serum.

There are various theories which fit these facts. An attractive one is that there is present in the sera effete polydisperse protein which the kidney is able to excrete by virtue of some vital activity. The existence of a vital activity which enables the kidneys to discriminate between homogeneous and heterogeneous protein would not be surprising in view of the fact that nature appears only to elaborate monodisperse proteins and these readily become polydisperse when subjected to artificial treatment. Denaturation and insolubility appear to be terminal stages in a state of progressively increasing molecular polydispersity. It would clearly be of importance therefore for the organism to be able to rid itself of polydisperse protein arising as the result of disease processes in the tissues.

Another explanation depends on the fact that many proteins are irreversibly altered by treatment with urea. Anson and Mirsky [1929], Wu [1929], Hopkins [1930], Pauli and Weiss [1931], and Burk [1932] have all investigated the effect of urea on proteins. A denaturation of the protein takes place which is apparent as an insolubility at the isoelectric point. Hopkins [1930] also obtains a positive nitroprusside reaction indicating constitutional changes in the protein molecule. Saturated or very concentrated solutions of urea are used to produce these effects. Hopkins finds that the proteins of diluted serum or of solutions of serum albumin are rendered insoluble much less readily than those of solutions of egg albumin, although they give the nitroprusside reaction at once after treatment with high concentrations of urea. This fact suggests that insolubility is a gross terminal stage of the effect and is preceded by less obvious chemical structural changes in the molecule. It may be therefore that lower concentrations of urea, such as are present in urine and in some pathological sera, are capable of causing an early partial denaturation of serum proteins which is only detectable by ultracentrifugal analysis. If this is the case it is possible that the polydispersity of the urinary and serum proteins which we have found is unrelated to the mechanism of protein excretion. Further ultracentrifugal investigation of the effect of moderate concentrations of urea on the serum proteins is required before we can come to a conclusion regarding this possibility.

In the two cases of our group which had globulin in the urine, *viz.* K.J.K. and L.E., the first was a characteristic case of chronic nephritis and the other was in an advanced state of toxaemia with a high probability of renal damage. In the other three which had no globulin in the urine there were no clinical grounds for suspecting renal disease. These facts suggest that when there is globulin in the urine the kidneys are damaged, and when the urinary protein gives only an albumin precipitation reaction we are concerned with polydisperse protein excreted by normal kidneys.

SUMMARY.

1. Five specimens of urinary proteins have been investigated in the ultracentrifuge. Two of these contain albumin and globulin fractions while three contain only albumin.

2. The albumin in all five specimens has an abnormally high power of boundary spreading, which suggests that the protein is wholly or partly polydisperse. The sera from the same five patients show similar signs of a polydisperse albumin fraction.

REFERENCES.

Anson and Mirsky (1929). *J. Gen. Physiol.* **13**, 1.
Burk (1932). *J. Biol. Chem.* **98**, 353.
Cavett and Gibson (1931). *J. Clin. Invest.* **10**; 857.
Forbes, Perlzweig and Burwell (1933). *South. Med. J.* **26**, 361.
Hewitt (1927). *Biochem. J.* **21**, 216.
—— (1929). *Lancet*, i, 66.
Hopkins (1930). *Nature*, **126**, 328, 383.
Kerridge (1934). *Lancet*, i, 675.
—— and Bayliss (1932). *Lancet*, ii, 787.
Kozawa, Iwatsuru and Adachi (1933). *Biochem. Z.* **260**, 313.
Mainzer (1932, 1). *Biochem. Z.* **246**, 134.
—— (1932, 2). *Biochem. Z.* **246**, 149.
—— (1932, 3). *Biochem. Z.* **246**, 156.
McFarlane (1935, 1). *Biochem. J.* **29**, 407.
—— (1935, 2). *Biochem. J.* **29**, 660.
—— (1935, 3). *Biochem. J.* **29**, 1175.
Pauli and Weiss (1931). *Biochem. Z.* **233**, 381.
Svedberg and Sjögren (1929). *J. Amer. Chem. Soc.* **51**, 3594.
Widdowson (1933). *Biochem. J.* **27**, 1321.
Wu (1929). *Chinese J. Physiol.* **3**, 1.

CXLVIII. THE ULTRACENTRIFUGAL ANALYSIS OF NORMAL AND PATHOLOGICAL SERUM FRACTIONS.

By ARTHUR SPROUL McFARLANE[1].

From the Institute of Physical Chemistry, Upsala, Sweden.

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In this paper, which is the concluding one of the series, we are concerned with the fractionation, by means of a neutral electrolyte and electrodialysis, of a few sera which have already been investigated in the ultracentrifuge [McFarlane, 1935, 1, 2, 3]. The particular objects in view have been (1) to investigate the composition of the globulin fractions obtained by a single precipitation with ammonium sulphate; (2) to compare the analytical results of the fractionation procedures with the results of ultracentrifugal analysis of the same serum; and (3) to discover in the case of certain sera whether new molecular types which have been shown to be present are precipitated or remain in solution on half saturation with ammonium sulphate.

For several reasons we have relied on ammonium sulphate as protein precipitant. It was advisable to use only one neutral electrolyte in order to be able to compare the results for one serum with those for another. Ammonium sulphate has been the most widely used electrolyte in the past in the fractionation of sera, although sodium sulphate is becoming increasingly used as a result of the work of Howe [1923]. It may be noted, however, that Howe finds the values for the albumin and globulin concentrations in cow sera obtained by a single precipitation with ammonium sulphate to agree with those obtained with sodium sulphate. Wu [1922] obtains the same analytical results with saturated magnesium sulphate as with half-saturated ammonium sulphate. Cullen and Van Slyke [1920] and Sørensen [1925; 1930] and others have used ammonium sulphate extensively in fractionating serum proteins.

It has been shown [1935, 1] that when horse serum albumin and globulin, prepared by ammonium sulphate fractionation, are mixed together in concentrated solution and the mixture is analysed in the ultracentrifuge, the concentration of the albumin fraction is found to be abnormally high and that of the globulin fraction correspondingly low. These proteins exist in concentrated solution in a form of equilibrium with each other. The same has been shown indirectly to be true for cow and human serum proteins by ultracentrifugal analysis of the concentrated and diluted sera. Since we know very little about the action of ammonium sulphate as a protein precipitant and in view of this difference in the state of the proteins in concentrated and diluted sera, it was decided in the first place to investigate the composition of the globulin precipitates obtained by half saturating a concentrated and a diluted cow serum with ammonium sulphate.

It is not proposed to repeat here the numerous details of the technique of an ultracentrifugal experiment which will be found in the earlier papers referred to. In the following tables we show the concentrations of the various protein fractions in g./100 ml. calculated from measurements of protein refraction increment using the values given in Table I of a previous paper [1935, 2] for the specific refraction increments (α) of the proteins. As already pointed out some of these α values cannot be regarded as generally established, and our concentration values may therefore be subject to errors. They are the best available α values and it serves a useful purpose here to record even approximate values for the protein concentrations.

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[1] Beit Memorial Research Fellow.

Exp. 1. Total globulins from normal cow and horse sera by precipitation with ammonium sulphate.

We employed in this experiment a portion of the cow serum used in Exps. 1–11, and a portion of the horse serum used in Exps. 12, 13, 15 and 16 of a previous paper [1935, 2]. The cow serum was treated in the following ways.

(*a*) 4 ml. of serum were diluted with 40 ml. of 1 % sodium chloride in a stoppered centrifuge-tube. 44 ml. of saturated ammonium sulphate were then added slowly and with constant shaking. The mixture was left for 2 hours during which time the globulin precipitate settled to the bottom. The tube was then centrifuged until the precipitate was well packed down on the bottom and the supernatant fluid could be poured off completely and the precipitate drained. This globulin precipitate was dissolved in water and dialysed against 1 % sodium chloride under pressure until equilibrium was reached. The solution (1·0 ml.) was transferred to a small graduated cylinder and the small collodion sac washed out with drops of dialysate which were transferred to the cylinder to make a final volume of 2·2 ml.

Results.

Refraction increment of globulin	0·01060
Therefore refraction increment of globulin in original serum ...	0·000583
Total protein refraction increment of original serum	0·01414
Therefore proportion of total protein refraction increment due to globulin fraction	41·2 %

(*b*) To 4 ml. of serum were added slowly and with shaking 4 ml. of saturated ammonium sulphate solution. The mixture was left for 2 hours and then centrifuged. The supernatant fluid was poured off and the precipitate drained and then stirred up with 8 ml. of saturated ammonium sulphate. After one hour the precipitate was again separated by centrifuging, suspended in a little water and dialysed against 1 % sodium chloride. The volume of combined protein solution and washings from the dialysis sac was in this case 3·0 ml.

Results.

Refraction increment of globulin	0·00818
Therefore refraction increment of globulin in original serum ...	0·00614
And proportion of total protein refraction increment due to globulin fraction	43·4 %

It is thus seen that the difference in the analytical results by these two procedures is very small.

The horse serum was treated as described under method (*a*) and the results were:

Volume of globulin solution	2·50 ml.
Refraction increment of globulin	0·01021
Therefore refraction increment of globulin in original serum ...	0·00638
Total protein refraction increment of original serum	0·01310
Therefore proportion of total protein refraction increment due to globulin fraction	48·7 %

Table I shows the results of the ultracentrifugal experiments on these three specimens of globulin. Fig. 1*a* shows the curve of cow serum globulin precipitated from dilute solution (exposure taken 57½ minutes after reaching full speed), and Fig. 1*b* shows the curve of horse serum globulin prepared in a similar way (exposure taken 116½ minutes after reaching full speed). The curves at corresponding times for cow serum globulin precipitated from concentrated and dilute solutions are all closely similar and differ markedly from the curves for horse serum globulin precipitated from dilute solution. In both specimens of cow serum globulin 30–35 % of other molecules (mainly *A* fraction) are present. All of this proportion, of course, does not represent albumin which we might hope to recover by repeated fractionation under ideal circumstances because the globulin was examined in concentrated solution. In such circumstances the

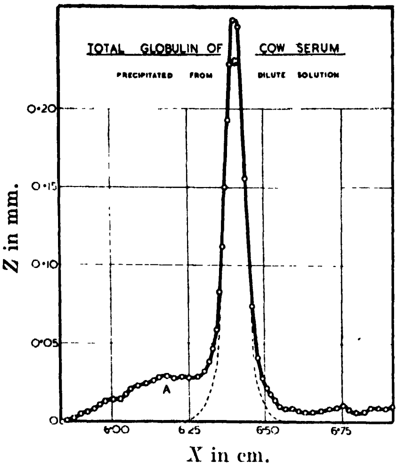


Fig. 1*a*.

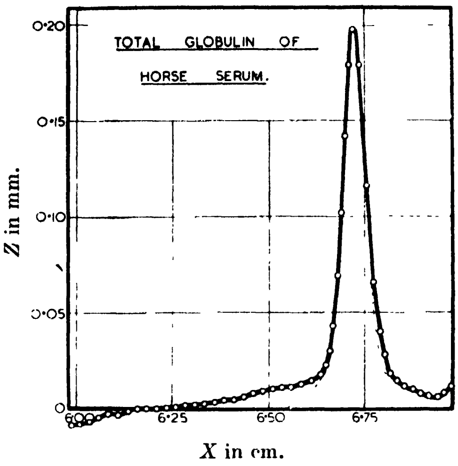


Fig. 1*b*.

Table I. *Results obtained in the investigation of total globulin from cow and horse sera.*

		Cow serum globulin precipitated from dilute solution	Cow serum globulin precipitated from concentrated solution	Horse serum globulin precipitated from dilute solution
Cell thickness (mm.)		2·00	2·00	2·00
Optical scale distance (cm.)		2·58	2·44	1·09
Cell temperature (first exposure)		33·8°	32·4°	31·5°
Cell temperature (last exposure)		34·3°	35·5°	34·3°
Approximate duration of run (hours)		2	2	2
Mean centrifugal force used (times gravity)		255,000	250,000	230,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$		0·0106	0·00818	0·0102
Corresponding total protein concentration (g./100 ml.)		5·65	4·42	5·48
Total $n_1 - n_0$ corrected to $\lambda = 366\,m\mu$		0·0114	0·00884	0·0110
Average $S_{W,20} \cdot 10^{13}$ of globulin		6·23	6·18	5·31
Average $n_1 - n_0$ from sedimentation diagram, $\lambda = 366\,m\mu$	Globulin	0·00560	0·00560	0·00833
	Other molecules	0·00309	0·00254	0·00089
	Total	0·00869	0·00814	0·00922
"% diff." total $n_1 - n_0$		−24 %	−8 %	−16 %
Proportion of total calculated $n_1 - n_0$ due to globulin fractions		64·5 %	69 %	90 %

concentration of the *A* fraction in a mixture is greater than corresponds to the albumin added. We have examined concentrated globulin solutions because these afford better possibilities of detecting traces of albumin.

Although the horse globulin was examined in more concentrated solution than the two cow globulin preparations the curves (*e.g.* Fig. 1*b*) show the presence of only some 10 % of an *A* fraction. From our experience with artificial mixtures of horse serum albumin and globulin [1935, 1] in various concentrations we estimate that at least 5 % of "recoverable" albumin is present in this preparation. Similarly, it is probable that some 15–20 % of recoverable albumin is present in both cow globulin preparations.

It is interesting to find so little difference in the two cow globulin preparations. We deduce from this experiment that when ammonium sulphate precipitates globulin from solution it is a matter of little importance whether all the globulin is present with the usual molecular dimensions, or is present, as in native sera, in a low molecular form probably in some way associated with albumin. Presumably, immediately the *G* fraction is precipitated from a concentrated mixture the equilibrium between *A* and *G* fractions results in the production of more *G* fraction which is precipitated, and this goes on until only albumin remains in the solution.

The preparation of horse serum globulin is surprisingly homogeneous for a single precipitation, in view of the fact that a preparation from another serum [*cf.* 1935, 1, Fig. 2*d*] after repeated reprecipitation contained nearly 20 % of an *A* fraction.

Exp. 2. Total globulins from normal human sera by precipitation with ammonium sulphate.

In this experiment we used the sera from O.D.B. and C.A. [1935, 2, Table XVII] and fractionated in accordance with method (*a*) above.

Results.

4 ml. of serum from O.D.B. gave 3·13 ml. of globulin solution.	
Refraction increment of globulin	0·00615
Therefore refraction increment of globulin in original serum ...	0·00482
And proportion of total protein refraction increment due to globulin fraction...	33 %
4 ml. of serum from C.A. gave 2 ml. of globulin solution.	
Refraction increment of globulin	0·00815
Therefore refraction increment of globulin in original serum ...	0·00407
And proportion of total protein refraction increment due to globulin fraction...	25·5 %

The results are shown in Table II and Fig. 2*a* shows the curve of an exposure taken 65 minutes after reaching full speed in the experiment on O.D.B.'s globulin, and Fig. 2*b* that of an exposure taken 69½ minutes after reaching full speed in the experiment on C.A.'s globulin. It is clear from these curves that both globulin preparations contain considerable amounts of albumin. It is calculated from the area of the curves that O.D.B.'s globulin contains no less than 43 % of an *A* fraction and C.A.'s contains 22 % of an *A* fraction.

It would clearly have been informative to examine the globulin preparations in a highly diluted state in order to determine how much of the *A* fraction represents theoretically recoverable albumin. This we have not done because the graphical analysis of the curves of very dilute mixtures is not accurate, particularly when we are concerned with a mixture containing excess of globulin. The data from the examination of these concentrated globulin solutions suffice to show, however, that the results of serum analysis which depend on a single precipitation with ammonium sulphate are not consistent. Heterogeneous globulin fractions result from the precipitation, and the degree of heterogeneity appears to vary not only between sera of different species but also between normal members of the same species. Results given later in this paper lend further support to these conclusions.

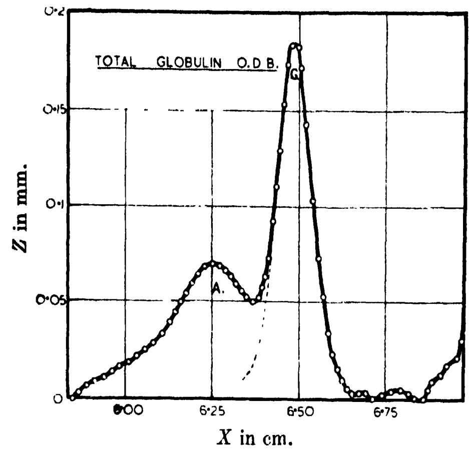


Fig. 2a.

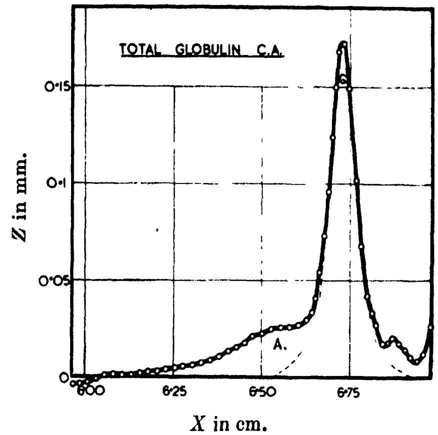


Fig. 2b.

Table II. *Results obtained in the investigation of the total globulins from the sera of O.D.B. and C.A.*

		Total globulin from serum of O.D.B.	Total globulin from serum of C.A.
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		5·28	1·98
Cell temperature (first exposure)		34·1°	32·4°
Cell temperature (last exposure)		34·5°	36·0°
Approximate duration of run (hours)		2	2
Mean centrifugal force used (times gravity)		255,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$		0·00615	0·00815
Corresponding total protein concentration (g./100 ml.)		2·81	3·72
Total $n_1 - n_0$ corrected to $\lambda = 366\, m\mu$		0·00664	0·00880
Average $S_{W,20} \cdot 10^{13}$ of globulin		6·22	5·87
Average $n_1 - n_0$ from sedimentation diagram, $\lambda = 366\, m\mu$	Globulin	0·00306	0·00546
	Other molecules	0·00229	0·00152
	Total	0·00535	0·00698
"% diff." total $n_1 - n_0$		−19·5 %	−21 %
Proportion of total calculated $n_1 - n_0$ due to globulin		57 %	78 %

It appears that some additional factor or factors other than the concentrations of the fractions determine whether ammonium sulphate produces a globulin precipitate which is free from albumin. It is well known that the results of the precipitation analysis of sera vary with the hydrogen ion concentration of the mixture. No doubt this is due to variable quantities of albumin which are precipitated with the globulin. It is also noteworthy in connection with the gross heterogeneity of the above normal human globulin preparations that Roche and Bracco [1934] found much lower molecular weights (103,000) for human globulin than for horse globulin (150,000) by osmotic pressure measurements.

Exp. 3. Serum fractions after drying.

We have described previously [1935, 2] the investigation of a human serum which had been dried by the Hardy-Gardner procedure using ether and alcohol at low temperatures. Much work has been done using albumin and globulin preparations obtained from this redissolved powder, and it seemed to us that by repeated fractionation it might be possible to obtain albumin and globulin fractions which were free from the polydisperse matter which we have shown to contaminate the dried serum. A dilute solution of the serum powder was therefore treated with an equal volume of saturated ammonium sulphate and the globulin precipitate separated off and reprecipitated twice from dilute solution. The precipitate was also washed once with half saturated ammonium sulphate solution between each precipitation. The albumin fraction was precipitated three times in all using saturated ammonium sulphate. The albumin and globulin solutions after dialysis against 1 % sodium chloride were examined in the ultracentrifuge and the results are shown in Table III.

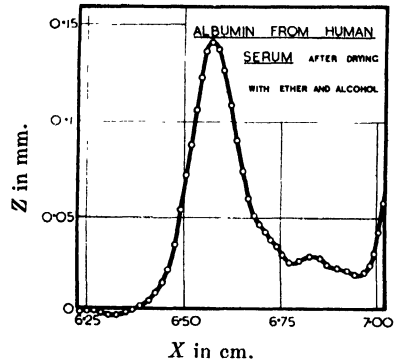


Fig. 3*a*.

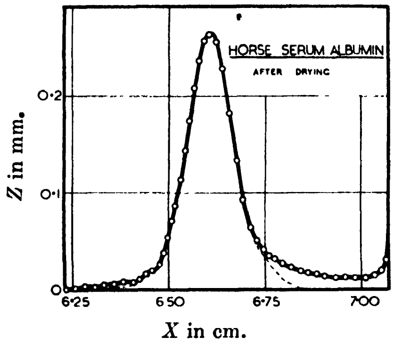


Fig. 3*b*.

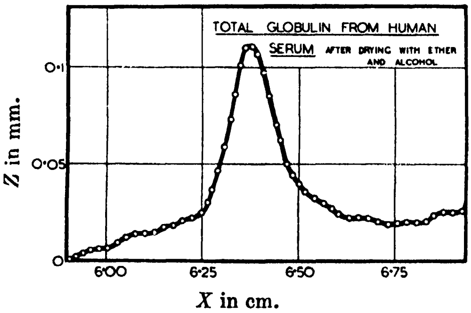


Fig. 3*c*.

Fig. 3*a* shows the curve of an exposure from the albumin experiment obtained 37½ minutes after reaching full speed. It is clear that considerable amounts of polydisperse protein still contaminate this protein. The curves of the globulin preparation show the presence of even greater amounts of polydisperse protein. We show a curve from this experiment in Fig. 3*c* obtained from an exposure taken 30 minutes after reaching full speed. From the curves it is calculated that less than 30 % of the total protein may be regarded as homogeneous globulin.

Table III. *Results obtained in the investigation of serum fractions which had been subjected to certain drying procedures.*

	Prepared from human serum which had been dried with ether and alcohol		Horse serum albumin after drying by simple evaporation
	Albumin	Globulin	
Cell thickness (mm.)	2·00	3·00	4·00
Optical scale distance (cm.)	4·44	5·64	3·64
Cell temperature (first exposure)	32·9°	32·1°	31·4°
Cell temperature (last exposure)	34·9°	34·9°	34·3°
Approximate duration of run (hours)	2	2	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	255,000	255,000	250,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$	0·00457	0·00381	0·00339
Corresponding total protein concentration (g./100 ml.)	2·28	1·74	1·85
A. Total $n_1 - n_0$ corrected to $\lambda = 366\, m\mu$	0·00494	0·00411	0·00366
Average $S_{W,20} \cdot 10^{13}$	4·18	6·66	4·23
B. Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\, m\mu$	0·00283	0·00127	0·00319
$\left(\frac{A-B}{B}\right) \times 100$	43 %	69 %	15 %
Boundary spreading coefficient (cm.^{2}/sec. 10^7)	5·9	9·2	4·8

We show in parallel (Table III) the results of an experiment on a recrystallised preparation of horse serum albumin which had been dried by simple evaporation under reduced pressure and then redissolved. The dried albumin, unlike the serum dried with ether and alcohol, did not redissolve completely. The experiment is also not an exact parallel with the earlier one because we have used horse instead of human albumin, and because in one case we are dealing with a dried albumin preparation whereas in the other we are dealing with an albumin fraction from dried whole serum. The results, however, together with the curve of Fig. 3*b* (from an exposure taken 40 minutes after reaching full speed) serve to illustrate an important fact which we have already recorded for dried cow serum, *viz.* that the soluble protein after drying by this technique has all the normal characteristics of the original protein.

Exp. 4. Fractions arising in the cataphoresis of normal human serum.

We have not obtained by any fractionation process a specimen of human serum albumin comparable in homogeneity with specimens of recrystallised horse serum albumin. Nor have we obtained a specimen of human globulin so nearly homogeneous as the best horse globulin preparations obtained by repeated precipitation [1935, 1] although these are not perfect in this respect. We therefore decided to try the method of transport cataphoresis and in this experiment we used an apparatus described by Theorell [1934][1]. About 100 ml. of fresh serum from a healthy student volunteer were placed in the bend of the special U-tube, the limbs of which are built up of short glass sections. By sliding shutters it is possible to close off the ends of each section without leakage so that each limb becomes subdivided into a number of closed cylindrical compartments and the fluid may be withdrawn from each compartment. Liquid junctions in the two limbs between the serum below and a phosphate buffer of the same p_H (7·21) and conductivity above were carefully made and cataphoretic movement of the serum protein induced in the usual way by means of a high potential applied through silver/silver chloride electrodes. The serum pigment migrated with the

[1] I am indebted to Med. Kand. O. Mellander for assistance in this experiment.

more rapidly moving albumin boundary. After 12 hours the various compartments were shut off and the solutions withdrawn, and a portion of each was tested for albumin and globulin. The solution from the upper compartment in the positive limb of the U-tube gave only an albumin reaction. The solutions from the upper three compartments in the negative limb gave no protein reactions, and that from the fourth gave only a globulin reaction. These albumin and globulin solutions were then dialysed against 1 % sodium chloride to determine the protein refraction increments and were then examined in the ultracentrifuge.

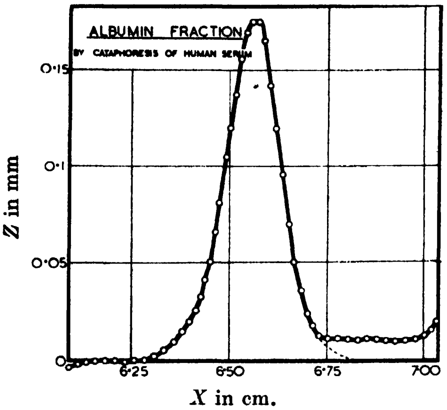


Fig. 4a.

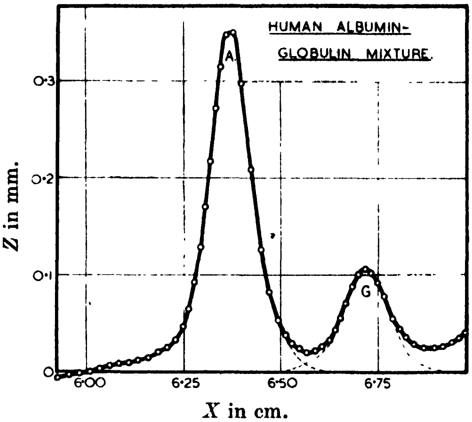


Fig. 4b.

Table IV. *Results obtained in the investigation of fractions arising in the cataphoresis of human serum.*

	Prepared from normal human serum by transport cataphoresis	
	Albumin	Globulin
Cell thickness (mm.)	4·00	3·00
Optical scale distance (cm.)	4·94	3·64
Cell temperature (first exposure)	32·8°	31·7°
Cell temperature (last exposure)	35·1°	34·1°
Approximate duration of run (hours)	2	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	250,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$	0·00189	0·00371
Corresponding total protein concentration (g./100 ml.)	0·95	1·70
Total $n_1 - n_0$ corrected to $\lambda = 366\,m\mu$	0·00204	0·00401
Average $S_{W,\,20} \cdot 10^{13}$	4·39	6·75
Average $n_1 - n_0$ from curve of sedimentation diagram $\lambda = 366\,m\mu$	0·00184	0·00289
"% diff." $n_1 - n_0$	-10 %	-28 %
Spreading coefficient of boundary (cm.^{2}/sec.$\cdot 10^7$)	6·3	4·9

Table IV shows the results of the two experiments, and Fig. 4*a* a curve from the albumin experiment from an exposure taken 55 minutes after reaching full speed. The curves show that the albumin contains only very small amounts of globulin, if any. It is the most nearly homogeneous human albumin preparation which we have obtained and suggests that the method of transport cataphoresis has considerable possibilities in the preparation of pure serum albumins where these are not crystallisable. It should be noted in particular from Table IV that the boundary spreading coefficient of the albumin corresponds well with the value for recrystallised horse serum albumin in corresponding concentration [*cf.* 1935, 1, Fig. 4]. The curves from the globulin solutions are also satisfactory and show no signs of an *A* fraction such as contaminates the human globulin preparations of Figs. 2*a* and 2*b*. The discrepancy between measured and calculated total $n_1 - n_0$ is rather high in this case. We conclude from this experiment that the cataphoresis method is on the whole a satisfactory one for fractionating human sera.

The object of this experiment was to obtain human albumin and globulin preparations which could be mixed together again and the mixture examined in the centrifuge. We obtained just sufficient of the above solutions to make a mixture of 3·57 g./100 ml. total protein concentration, and we show in Fig. 4*b* a curve of this mixture obtained from an exposure taken 55 minutes after reaching full speed. From the curves of this experiment we calculate the albumin/globulin ratio to be 2·9. The ratio of the quantities of albumin to globulin added was 1·3. Even if we regard the globulin preparation as only 80 % monodisperse it is clear that on mixing the two together a considerable shift has occurred in the ratio of the fractions. From this experiment it is deduced that the equilibrium between the fractions in human serum is reversible just as we have shown it to be in the case of horse serum fractions. It is, however, not reversible in one respect. It will be noted that the *A* curve in Fig. 4*b* is quite symmetrical, no signs of an *X* fraction being detectable. We have noted [1935, 2] the same irreversible change when cow and horse sera are treated with ammonium sulphate. The significance of the phenomenon is obscure.

Exp. 5. Globulin fractions from antidiphtheritic horse serum.

It will have been noted in the examination of the total globulins of normal sera that no distinct molecular types exist in these which might correspond to the pseudoglobulin and euglobulin fractions. If these two fractions represent different proteins we must conclude either that the two have the same molecular weight but different chemical constitutions, or that they are produced by the process of electrodialysis which leads to their separation. To investigate this matter we prepared the total globulin from an immune serum[1] [1935, 3, Exp. 1, Serum 2] by one precipitation with ammonium sulphate and electrodialysed a portion of this to give pseudoglobulin and euglobulin fractions.

Results.

10 ml. of serum 2 gave 16·34 ml. of globulin solution.

Refraction increment of globulin	0·00833
Therefore refraction increment of globulin in original serum ...	0·01361
Total protein refraction increment of original serum	0·01770
Therefore proportion of total protein refraction increment of original serum due to total globulin fraction	77 %

It may be mentioned here incidentally that antidiphtheritic serum 1 [1935, 3] when subjected to the same analytical procedure gave a globulin proportion of 79·5 %.

The total globulin of serum 2 was centrifuged and Fig. 5*a* shows the curve of an exposure taken 50 minutes after reaching full speed. It will be seen from this and from the results in Table V that the preparation is nearly homogeneous

[1] The fractionation of immune sera into eu- and pseudo-globulin fractions is now a process of considerable commercial importance.

which is surprising for a single precipitation. We may assume that the proportion of homogeneous globulin in the original serum is slightly less than the analytical figure—say 75 %—on account of traces of albumin in the globulin preparation. Ultracentrifugal analysis of the undiluted serum [1935, 3] showed that exactly equal amounts of *A* and *G* fractions were present. This means that the area of the *A* curve corresponds to twice the amount of albumin which is recoverable from this serum and the area of the *G′* curve to two-thirds the recoverable globulin. These figures give some idea of the extent to which the albumin/globulin ratio in an untreated immune serum may be altered by the equilibrium between the fractions.

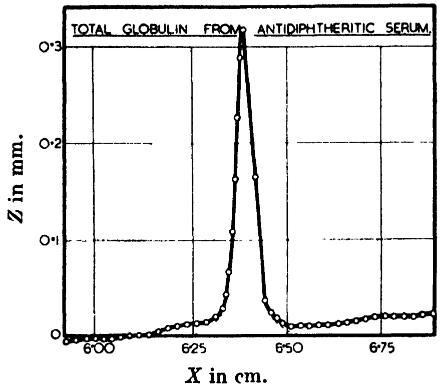


Fig. 5*a*.

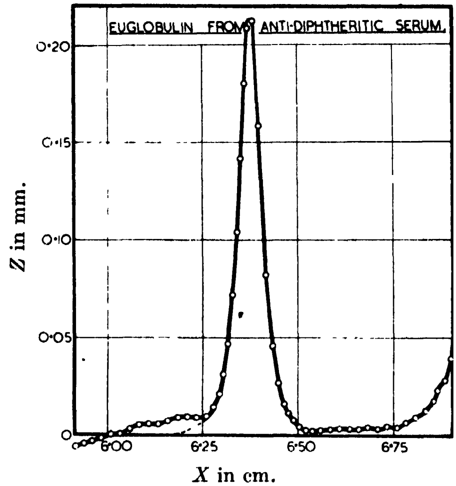


Fig. 5*b*.

Table V. *Results obtained in the investigation of globulin fractions from an immune serum.*

	Fractions from antidiphtheritic horse serum		
	Total globulin	Pseudo-globulin	Euglobulin
Cell thickness (mm.)	2·00	2·00	2·00
Optical scale distance (cm.)	1·38	1·68	2·28
Cell temperature (first exposure)	34·8°	33·0°	32·7°
Cell temperature (last exposure)	35·2°	35·5°	34·9°
Approximate duration of run (hours)	2	$1\frac{3}{4}$	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	255,000	255,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$	0·00833	0·00619	0·00915
Corresponding total protein concentration (g./100 ml.)	4·48	3·33	4·92
Total $n_1 - n_0$, corrected to $\lambda = 366\,m\mu$	0·00899	0·00668	0·00988
Average $S_{W,20} \cdot 10^{13}$	5·81	6·31	6·16
Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\,m\mu$	0·00788	0·00603	0·00499
"% diff." $n_1 - n_0$	−12·3 %	−9·7 %	−49·5 %
Spreading coefficient of boundary (cm.^{2}/sec. × 10^7)	0·15	0·36	1·3

10 ml. of the total globulin of serum 2 were dialysed at 4° against distilled water and then electrodialysed according to the procedure described by Ettisch and Ewig [1928]. As recommended by Ettisch and de Loureiro [1933] we have

used at the cathode a membrane of parchment and at the anode a membrane of glycine-collodion. The electrodialysis was completed inside an hour with no detectable rise of temperature in the cell. The liquid in the centre compartment was stirred up and poured off. The precipitate was separated by centrifuging, drained and stirred up with 1 % sodium chloride in which it redissolved slowly and incompletely to an opalescent solution. After standing overnight in contact with the insoluble globulin the solution of euglobulin was dialysed against 1 % sodium chloride and examined in the ultracentrifuge. The pseudoglobulin solution was dialysed against 1 % sodium chloride and similarly examined. The results are shown in Table V, and Fig. 5*b* shows the curve of an exposure taken 43 minutes after reaching full speed in the experiment on the euglobulin fraction. The pseudoglobulin fraction differs in no significant details from the original total globulin preparation. The euglobulin preparation, on the other hand, is largely polydisperse, as is shown by the shape of the curves, the large "% diff." values, and the abnormally large boundary spreading coefficient. Svedberg and Sjögren [1930] and Mutzenbecher [1931] found both the pseudoglobulin and euglobulin of normal horse sera to be polydisperse, the former fraction much less so than the latter. Svedberg and Sjögren did not use differently charged membranes and their electrodialysis lasted for 36 hours. Mutzenbecher used the same technique as that described above but electrodialysed whole serum instead of total globulin. These differences may explain why our pseudoglobulin preparation appears to be more nearly homogeneous than theirs. Alternatively, antidiphtheritic horse serum may be capable of giving a relatively homogeneous pseudoglobulin fraction while normal horse serum is not. We may say at least that the polydisperse nature of euglobulin seems to be fully substantiated. Our experiment also shows that the pseudoglobulin of antidiphtheritic horse serum does not contain any homogeneous fraction of significantly different molecular weight from that of the total globulin.

Exp. 6. Arbitrary globulin fractions from the serum of G.N. *(septic pneumonia).*

It has been shown in Exp. 8 of a previous paper [1935, 3] that in the untreated state the protein of G.N.'s serum is approximately equally distributed between *A*, *G* and *X* fractions. We employed for this serum a purely arbitrary method of fractionation. 4·24 ml. of serum were diluted with 40 ml. of 1 % sodium chloride, and saturated ammonium sulphate was added with shaking until a substantial precipitate had appeared as judged by the turbidity of the solution. This required 23 ml. of the ammonium sulphate solution. The precipitate was centrifuged off, dialysed and examined in the ultracentrifuge (fraction 1). More saturated ammonium sulphate (4 ml.) was added until another substantial precipitate had appeared and this was also separated and dialysed (fraction 2). Sufficient ammonium sulphate (17·24 ml.) was now added to make half saturation and the remainder of the globulin separated off (fraction 3). From the results of the refractive index measurements we obtain the following values for the proportions of the total refraction increment of the original serum which are due to each fraction.

Fraction 1	15 %
,, 2	16 %
,, 3	30·5 %

The results of the ultracentrifugal examination of these three fractions are shown in Table VI, and Fig. 6*a* shows the curves of an exposure taken 56 minutes after reaching full speed in the experiment on fraction 2. The results for the three globulin fractions indicate that these have approximately the same composition, if we except differences in the "% diff." values which indicate mainly differences in the amount of polydisperse protein in each fraction. It is interesting to find also that the curves of all three fractions show very small proportions of *A* fractions to be present—much less than we have found in the globulin fractions of normal human sera. These erratic results all support the view already stated that some factor—probably electrochemical—of which we have no precise knowledge determines the ultimate degree of homogeneity of the globulin precipitates obtained by treating serum with ammonium sulphate.

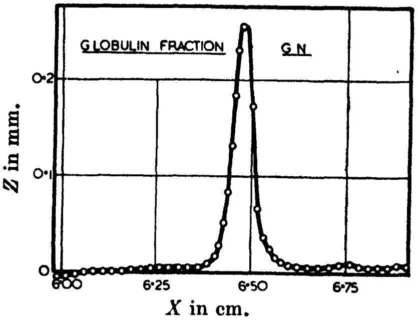


Fig. 6.

Table VI. *Results obtained in the investigation of certain arbitrary globulin fractions from a pathological human serum (G.N.).*

	Globulin fraction (1)	Globulin fraction (2)	Globulin fraction (3)
Cell thickness (mm.)	2·00	2·00	2·00
Optical scale distance (cm.)	2·14	1·68	1·08
Cell temperature (first exposure)	32·6°	33·9°	29·6°
Cell temperature (last exposure)	35·5°	36·1°	33·0°
Approximate duration of run (hours)	$1\frac{3}{4}$	$1\frac{3}{4}$	$1\frac{3}{4}$
Mean centrifugal force used (times gravity)	250,000	255,000	245,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\, m\mu$	0·00687	0·00728	0·00875
Corresponding total protein concentration (g./100 ml.)	3·14	3·32	4·00
Total $n_1 - n_0$, corrected to $\lambda = 366\, m\mu$	0·00742	0·00786	0·00945
Average $S_{W,20} \cdot 10^{13}$	6·18	6·20	5·65
Average $n_1 - n_0$ from curve of sedimentation diagram, $\lambda = 366\, m\mu$	0·00578	0·00680	0·00665
"% diff." $n_1 - n_0$	−22 %	−13·5 %	−29·5 %
Spreading coefficient of boundary (cm.²/sec. × 10⁷)	0·26	0·27	0·18

Exp. 7. Total globulin fractions from the sera of A.J. (malignant disease) and G.L.E. (myeloma).

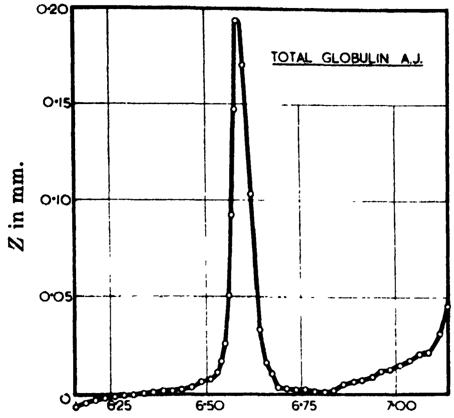
4 ml. of A.J.'s serum analysed by procedure (*a*) gave 2·0 ml. of a globulin solution.

Refraction increment of globulin	0·0106
Therefore refraction increment of globulin in original serum	0·00503
Total protein refraction increment of original serum	0·0128
Therefore proportion of total protein refraction increment of original serum due to globulin fraction	39·3 %

4 ml. of G.L.E.'s serum yielded 8 ml. of globulin solution.

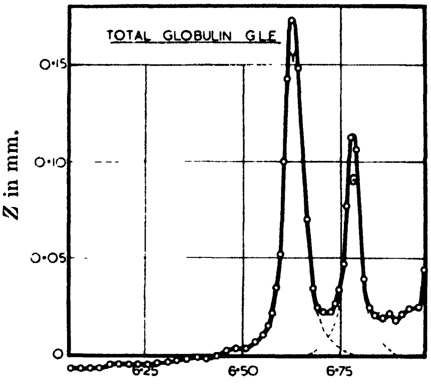
Refraction increment of globulin	0·00796
Therefore refraction increment of globulin in original serum	0·01592
Total protein refraction increment of original serum	0·0220
Therefore proportion of total protein refraction increment of original serum due to globulin fraction	72·4 %

The results are shown in Table VII, and Fig. 7*a* shows a curve of A.J.'s globulin (from an exposure taken 44½ minutes after reaching full speed), and Fig. 7*b* a curve of G.L.E.'s globulin (from an exposure taken 65 minutes after reaching full speed).



X in cm.

Fig. 7*a*.



X in cm.

Fig. 7*b*.

Table VII. *Results obtained in the investigation of the total globulins from the sera of A.J. (malignant disease) and G.L.E. (myeloma).*

	Total globulin from serum of A.J.	Total globulin from serum of G.L.E.
Cell thickness (mm.)	2·00	2·00
Optical scale distance (cm.)	0·78	1·38
Cell temperature (first exposure)	33·2°	31·1°
Cell temperature (last exposure)	35·7°	33·9°
Approximate duration of run (hours)	1¾	2
Mean centrifugal force used (times gravity)	255,000	255,000
Total n_1-n_0, by refractometer, $\lambda=589\,m\mu$	0·0106	0·00796
Corresponding total protein concentration (g./100 ml.)	4·84	3·64
Total n_1-n_0, corrected to $\lambda=366\,m\mu$	0·0115	0·00860
Average $S_{W,20}\cdot 10^{13}$	5·01	5·56 (Y) 6·77 (G)
Average n_1-n_0 from curve of sedimentation diagram, $\lambda=366\,m\mu$	0·00930	0·00443 (Y) 0·00247 (G)
Total n_1-n_0 from curve of sedimentation diagram, $\lambda=366\,m\mu$	0·00930	0·00690
"% diff." total n_1-n_0	−19 %	−20 %

The total globulin from A.J.'s serum appears to be almost completely free from any A fraction and except for the discrepancy of −19 % between calculated and measured refraction increments it has the characteristics of a homogeneous protein. It is also noteworthy in this experiment that the proportion of globulin fraction obtained by precipitation analysis, *viz.* 39·3 % is in exact agreement with the proportion obtained by ultracentrifugal analysis of the serum diluted with 4 volumes of 1 % sodium chloride.

Fig. 7*b* and the results in Table VII show that the globulin precipitate from G.L.E.'s serum is composed of two molecular types corresponding approximately in sedimentation constants to the Y and G fractions of the original serum. Both fractions appear to be homogeneous.

Exp. 8. Total albumin and total globulin fractions from the serum of K.M. (malignant disease).

In the earlier examination of this serum [1935, 3] we found that four molecular fractions were present and the evidence was in favour of the view that the lightest fraction is an abnormal type and the three heavier fractions correspond to the A, X and G fractions of normal sera. The serum was fractionated by the simplest possible procedure. The total globulin precipitate obtained according to procedure (*a*) was redissolved, dialysed and examined in the centrifuge. The mother-liquor from this precipitation was dialysed under pressure whereby all the ammonium sulphate was removed and the protein was obtained in concentrated solution. This solution was then examined in the centrifuge.

Results.

4 ml. of K.M.'s serum yielded 2·4 ml. of globulin solution.

Refraction increment of globulin	0·00662
Therefore refraction increment of globulin in original serum	0·00397
Total protein refraction increment of original serum	0·0118
Therefore proportion of total protein refraction increment of original serum due to globulin fraction	33·6 %

The results of the ultracentrifugal experiments are shown in Table VIII. The curve of Fig. 8*a* is from an exposure taken 100 minutes after reaching full speed in the experiment on the total albumin; that of Fig. 8*b* is from an exposure taken 46½ minutes after reaching full speed in the experiment on the total globulin. These curves and the results obtained from them demonstrate clearly what has happened to the new molecular type in the processes of fractionation. The new (A_1) molecule has remained in solution in close association with the A fraction. The ratio of the concentration of A_1 to A molecules in the albumin fraction is however markedly different from their ratio in the original serum. There are several possible explanations of this, but none that can be substantiated in the meantime.

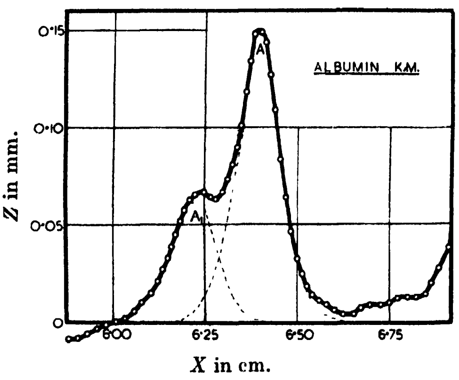


Fig. 8*a*.

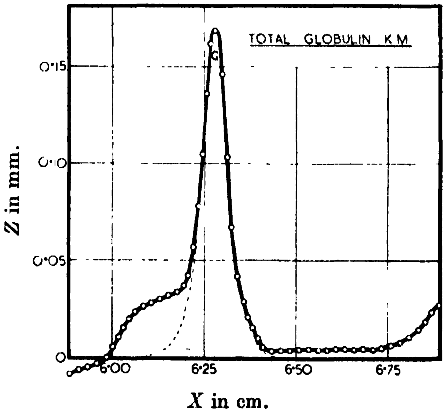


Fig. 8*b*.

The globulin fraction contains slightly more than 20 % of an A fraction. In this respect it differs from the preceding pathological globulin preparations and resembles the preparations from normal human sera (Exp. 2).

Table VIII. *Results obtained in the examination of the albumin and total globulin fractions from the serum of K.M. (malignant disease).*

		Albumin fraction	Total globulin
Cell thickness (mm.)		2·00	2·00
Optical scale distance (cm.)		3·18	2·28
Cell temperature (first exposure)		30·2°	30·9°
Cell temperature (last exposure)		35·8°	34·3°
Approximate duration of run (hours)		3	2
Mean centrifugal force used (times gravity)		250,000	255,000
Total $n_1 - n_0$, by refractometer, $\lambda = 589\,m\mu$		0·00659	0·00662
Corresponding total protein concentration (g./100 ml.)		3·30	3·02
Total $n_1 - n_0$, corrected to $\lambda = 366\,m\mu$		0·00712	0·00715
Average $S_{W,20}\cdot 10^{13}$		2·70 (A_1)	6·23 (G)
		3·71 (A)	—
Average $n_1 - n_0$ from sedimentation diagram, $\lambda = 366\,m\mu$	Fractions	0·00177 (A_1)	0·00410 (G)
		0·00458 (A)	0·00109 (A)
	Total	0·00635	0·00519
"% diff." total $n_1 - n_0$		-11 %	-27 %

GENERAL DISCUSSION.

The view that the albumin and globulin which can be isolated from serum represent stable chemical individuals probably existing as such in the original untreated serum has been widely accepted for many years. The popularity of the view is due to the agreement which has been obtained by many workers studying various physical and chemical properties of these proteins. Serum albumin has become generally regarded as an amphoteric substance of well-defined elementary chemical composition, molecular weight, isoelectric point, electrophoretic mobility, specific refraction increment, specific light absorption and optical rotation. When one considers the possibilities for differences between the results of various observers which are due to differences and errors in the techniques of observation, it is probably fair comment to say that the general measure of agreement in regard to the properties of serum albumin is satisfactory and in support of the view that all observers are examining essentially the same substance. The same cannot be said confidently of serum globulin, because in this case the measure of agreement among different observers has not been good. There has been, however, a strong undercurrent of belief in regard to this substance that the disagreements arise from the presence of quantities of contaminating substances, and that in the preparation of each observer there is present in greater or smaller concentration, a common substance.

Sørensen, to whose earlier work on egg albumin is due probably more than to anything else, the inception of the view that the proteins are stable chemical entities, has recently [1930] given good reasons for doubting the fundamental principles on which this view is based. It is now Sørensen's contention that the uniformity of the results obtained for the serum proteins is not a criterion of the eventual individuality and purity of these substances but only of the reproducibility of the physical and chemical conditions under which the proteins are prepared. He regards both proteins of serum as component or "co-precipitation" systems made up of stable main valency units (probably polypeptides) which are loosely held together by residual valencies. The ultimate size, number and general properties of these loosely constructed units is determined by the physical conditions which hold in the solution at any instant. Since practically all observers have obtained their albumin and globulin fractions from solutions of closely prescribed ammonium sulphate content it is to be expected that they should obtain fractions of similar composition.

Sørensen's conclusions would be more convincing to us if his carefully conducted experiments had been carried out with fresh untreated serum instead of with serum powder dried by the Hardy-Gardner procedure, and also if his proof had contained more definite physical evidence of the existence of his protein co-precipitation systems in native protein solutions. The facts which we have related in the foregoing series of papers, however, neither support nor disprove his theory because our proof of the existence of albumin and globulin fractions in mixtures over a range of concentrations is purely arbitrary. Our practice has been to find first an empirical relationship between the concentration and sedimentation constant of a protein, and thereafter to use the sedimentation constant to identify the protein in any concentration. The original relationship may depend on the effects of viscosity alone or on a combination of viscosity effects and changes in the molecular dimensions of the protein. The real test of Sørensen's theory is whether or not the observed molecular weights of the serum proteins tend to a constant value in dilute solutions. From a consideration of the data of Svedberg and Sjögren [1930] on the sedimentation equilibrium of serum proteins, and of Adair and Robinson [1930] on the osmotic pressure of the serum proteins we incline to the view that serum albumin at least has a constant molecular weight in high dilutions.

We can say from the results of this work that untreated sera or concentrated mixtures of the serum proteins do not contain a collection of residual valency units which are all of the same particle size. Sørensen does not say whether his co-precipitation systems might be expected to contain under a given set of conditions a small or a large number of different particle sizes. The ultracentrifuge shows that in normal serum there are present three fractions each of which if not completely homogeneous can only be heterogeneous within very narrow limits. We may assume with Sørensen that the serum represents a set of three co-precipitation systems, but we must postulate that specific molecular forces are operative in producing and maintaining only those three sets of loosely bound residual valency units. Sørensen's theory does not explain the nature of these forces.

In one important respect Sørensen's views coincide strikingly with the facts of our experiments. To quote his own words "an interaction must be assumed, for example in serum, between the protein systems mutually as well as between these and other substances present". The equilibrium which we have shown to exist between *A* and *G* fractions in serum appears to agree with his prediction in all essentials. It must be remarked, however, that this equilibrium phenomenon does not necessarily require that the *A* and *G* fractions separately should be reversibly dissociable systems. The phenomenon is equally well explained by the theory that two stable proteins are able to form loose combinations when present in sufficient concentration. Neither point of view offers any explanation of the really unusual fact about this equilibrium, *viz.* that the protein complex or compound (according to the viewpoint adopted) has lower molecular dimensions than at least one of the disruption systems (or stable proteins) to which it gives rise on dilution.

Whether Sørensen's view that the proteins as a class are reversibly dissociable polypeptide systems is true or not is of great importance in connection with the results of investigations on isolated proteins. On the other hand it has only a small significance for the results of investigations on untreated serum. In the latter case it is frequently only desired to know the nature of the protein fractions as they exist in serum and it is of minor importance whether these fractions represent proteins which may be isolated from the serum or dissociable systems of no fixed composition. In general, however, the prospect of enlarging our knowledge of the protein constitution of serum will be greatly increased if it is possible to separate the proteins in stable form and examine the pure solutions. For this reason we have conducted the preliminary fractionation investigations reported in this paper.

The outstanding feature of these few experiments is the lack of uniformity in the composition of the globulin precipitates obtained by half saturation with ammonium sulphate. Whereas certain sera, *e.g.* normal human and cow sera, gave rise to globulin precipitates which contained relatively large quantities of albumin, other sera, *e.g.* normal horse and some pathological human sera, gave rise to globulin fractions which contained only small amounts of albumin. The results of the latter experiments suggest that under certain conditions even a single precipitation with ammonium sulphate is capable of giving rise to satisfactorily homogeneous globulin preparations. The results of the experiments as a whole, however, suggest that we have still to find out what constitute ideal conditions for quantitatively fractionating serum by means of ammonium sulphate.

An experiment with the transport cataphoresis method of fractionation has indicated that there are possibilities inherent in this technique, particularly with regard to the fractionation of human sera. On theoretical grounds the method appears to be an ideal one for the preparation of serum proteins and it is hoped to attempt its application later to the isolation of the new molecular types which have been shown to exist in certain pathological sera.

We have examined the ammonium sulphate fractions from two pathological sera in which the evidence for the existence of new molecular types could be regarded as quite definite. The results indicate that in both cases the new molecular types have retained their individuality after the treatment with ammonium sulphate, a behaviour which does not seem to be shared by the *X* fraction of normal sera. This fact holds out considerable prospect that they represent stable proteins which like albumin and globulin are capable of being isolated in a comparatively pure state. The only satisfactory proof of this is, of course, actually to isolate them and demonstrate their purity. We anticipate, however, that many methods of fractionation will need to be tried and the results tested at every step by ultracentrifugal analysis before a suitable fractionation technique will be found.

SUMMARY.

Fractions obtained from various sera have been investigated in the ultracentrifuge.

1. The total globulin from a normal cow serum, whether precipitated from concentrated or dilute solution with ammonium sulphate, was found to be markedly heterogeneous due to the presence mainly of albumin.
2. The total globulins precipitated from two normal human sera in dilute solution with ammonium sulphate also contained quantities of albumin.
3. Albumin and globulin fractions were isolated from a human serum which had been dried with ether and alcohol. In spite of repeated precipitations with ammonium sulphate these fractions contained large amounts of polydisperse protein.

4. Albumin and globulin fractions obtained by cataphoresis of a normal human serum were found to be satisfactorily homogeneous.

5. The total globulin from an antidiphtheritic horse serum after one precipitation with ammonium sulphate contained only insignificant amounts of albumin. The pseudoglobulin obtained from this preparation by electrodialysis had approximately the same composition as the total globulin but the euglobulin was at least 50 % polydisperse.

6. In the fractionation of two pathological human sera in dilute solution by means of ammonium sulphate, homogeneous globulin fractions were obtained.

7. Two pathological sera in which the presence of new proteins had previously been demonstrated were fractionated by means of ammonium sulphate. In one case the new protein was found in the albumin fraction and in the other in the globulin fraction.

The general conclusion is drawn that a single precipitation with ammonium sulphate is not a satisfactory method of fractionating sera particularly for purposes of quantitative analysis.

It is a pleasure to acknowledge the unfailing interest and valuable advice of Prof. Svedberg in the course of this work and the generous hospitality afforded in his laboratory. To his assistants, particularly to Dr Kai O. Pedersen, I wish to express my great indebtedness. The expenses of this investigation have been defrayed by grants from the Andersson Foundation, the Nobel Foundation and the Rockefeller Foundation.

It will be possible to continue this work at the Lister Institute, London, where an ultracentrifuge now under construction in Upsala will be installed in the near future.

REFERENCES.

Adair and Robinson (1930). *Biochem. J.* **24**, 1864.
Cullen and Van Slyke (1920). *J. Biol. Chem.* **41**, 587.
Ettisch and Ewig (1928). *Biochem. Z.* **195**, 175.
—— and de Loureiro (1933). *Biochem. Z.* **266**, 422.
Howe (1923). *J. Biol. Chem.* **57**, 241.
McFarlane (1935, 1). *Biochem. J.* **29**, 407.
—— (1935, 2). *Biochem. J.* **29**, 660.
—— (1935, 3). *Biochem. J.* **29**, 1175.
Mutzenbecher (1931). *Biochem. Z.* **235**, 425.
Roche and Bracco (1934). *Compt. Rend. Acad. Sci.* **199**, 98.
Sørensen (1925). *Compt. Rend. Carlsberg Lab.* **15**, No. 11, 1.
—— (1930). *Compt. Rend. Carlsberg Lab.* **18**, No. 5, 1.
Svedberg (1930). *Kolloid Z.* **51**, 10.
—— and Sjögren (1930). *J. Amer. Chem. Soc.* **52**, 2855.
Theorell (1934). *Biochem. Z.* **275**, 1.
Wu (1922). *J. Biol. Chem.* **51**, 33.

CXLIX. DETERMINATION OF VITAMIN A WITH THE HILGER VITAMETER EQUIPPED WITH A DEVICE FOR PHOTOGRAPHIC RECORDING.

By OLAV NOTEVARP.

From the Norwegian Fisheries Research Station and Official Cod Liver Oil Control Laboratories, Bergen, Norway.

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The ultraviolet absorption at 328 $m\mu$ has now been generally accepted to be, with certain limitations, a measure of the content of vitamin A [League of Nations Commission, 1934]. The spectrographic determination of this, however, requires elaborate and expensive apparatus and a considerable amount of work by expert hands, which makes it unsuitable for use as a general method of analysis. For the Official Norwegian Cod Liver Oil Control Laboratories under technical leadership of the Fisheries Research Station, it was very desirable to be able to carry out the determination by means of some simpler apparatus, and as early as 1932 enquiries were made with the leading makers of optical instruments whether it was possible to manufacture a simple apparatus suitable only for measuring $E_{328\,m\mu}$. At that time only the usual spectrophotometers were available, but early in 1933 the firm A. Hilger, London, sent out their vitameter, which was exactly the instrument we were looking for, and the first one manufactured was delivered to our laboratories.

A description of the vitameter has been published by Adam Hilger, Ltd. The measurement, as far as the observer is concerned, consists of the matching of intensity of two fluorescent lines. We soon found that the matching of the intensity of the two fluorescent lines was difficult, determinations carried out at different times and by different observers giving discrepancies of up to ± 10–15 %. In order to avoid this I tried to record the intensity of the lines photographically, and it was found that they had sufficient photographic activity to give prints within a few seconds on the most vigorous gaslight printing paper available. This material has the great advantage of being independent of a dark-room, which makes developing *etc.*, a very simple matter.

I therefore equipped our vitameter with a simple device for photographing the lines on such paper, and this has been in constant use during the last year and a half and has enabled us to obtain exact and objective observations quickly and easily.

The construction of the device will be seen in Fig. 1. Immediately in front of the fluorescent screen there have been made two narrow slits to allow the insertion of a holder for the paper. This runs in rails that are fixed to the support for the screen, both holder and rails being made so as to get the paper as close as possible to the screen. The paper is held in place by the turned down edges of the holder and is pushed in till it meets a stop, to ensure that it is always held in the same place. On the back of the holder there are marks to show where to begin and how far it is to be moved for each exposure. We have found a size of paper 9 × 4 cm. suitable.

To carry out the determination a suitable solution is first prepared, which should have an extinction coefficient of 0·6–1·0. The absorption is first determined approximately by eye, a shutter is placed in front of the lens and the paper brought into position in the holder. It should be marked to show which side is up. Exposures are then made at suitable intervals of E, the holder being moved about 5 mm. between each exposure. The time necessary increases as the aperture is reduced; we have found a suitable exposure to be ten times as many seconds as the aperture reading ($E = \log I_0/I$) running the copper arc at 45–50 volts, 0·45 ampere, and with a gap of about 1 mm. The paper is then developed for one minute in an ordinary, vigorous paper developer, and fixed. This can be done in electric or subdued daylight if the paper is protected from direct rays. Even greater freedom can be obtained by using a desensitising bath first.

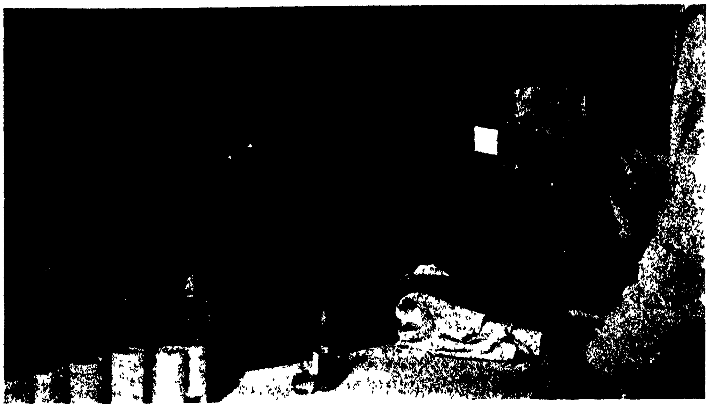
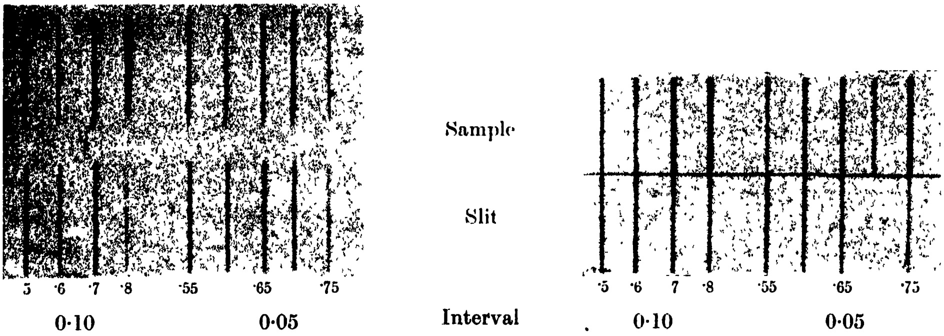


Fig. 1.



Original print.

Print with lines brought together to facilitate reading.

Fig. 2.

It will be necessary to expose from about 20 % below to 20 % above the approximate value, the intervals being chosen according to the accuracy desired. If the apertures have been correctly chosen it will be found that the print of the line that has passed through the solution is weaker than that from the aperture for the lowest values, the difference growing less until there is equality and then the case is reversed. A typical set of exposures is shown in Fig. 2.

The intervals may be chosen down to $0{\cdot}05E$ and it is possible to read with an accuracy of $\pm 0{\cdot}01$. Parallel determinations should not differ more than $\pm 0{\cdot}02$, that is $\pm 3\ \%$ if the observation is carried out at 0·6–0·7.

It will be seen that the accuracy is considerably greater than is obtainable by ocular readings and approaches the accuracy obtainable by usual spectrographic methods.

Corrections. The increased accuracy makes it necessary to take certain precautions. The glass cell must be perfectly clean and should be polished before each observation with a piece of clean wash-leather. The solutions must be free from any turbidity, and a correction must be introduced for the solvent used. This makes the choice of solvent unimportant as long as its absorption is low and it does not in any way harm the vitamin.

Table I gives some of the values found for various solvents in one of the cells that have been used. It should be noted that these cannot be regarded as absolute values of $E_{328\,m\mu}$. They may be expected to vary with different instruments and cells, as the latter and the compensating glass plates may differ slightly. No special precautions have been taken to purify the solvents, the chloroform is ordinary B.P. and the alcohol may be expected to contain aldehyde, as it has not been protected from air.

Table I. E *for solvents in vita-glass cell No. II.*

						Mean value
Chloroform	0·04	0·05	0·03	0·04	0·03	0·04
Alcohol, absolute	0·07	0·09	0·11	0·10	0·08	0·09
The same + 10 % chloroform	0·07	0·07	0·05	0·07	0·08	0·07
Toluene	0·07	0·07	0·08	—	—	0·07

Although it has a relatively great absorption, and dissolves only a limited quantity of oil, we have found that absolute alcohol is the most suitable solvent, as vitamin A is more stable in this than in other solvents, and it does not evaporate very quickly.

Setting of the vitameter. It is imperative that the arc should be placed exactly at the correct distance from the lens, as the changes in aperture are not symmetrical. The prints of the two lines will then appear exactly one above the other and be equally broad, and variations in density across the lines, if any, should correspond. If the arc is out of focus, the line from the aperture will be blackened unsymmetrically, and it is difficult to obtain correct readings. To set the vitameter it is best to use a solution with a density of 0·8–1·0. The arc is then set and the lines matched as nearly as possible by eye. Exposures are made with the arc at slightly different distances (2–4 mm.), it being then easy to see from the prints which is the right distance. The setting can be checked by determining the same substance in several concentrations. Table II gives some values of the same oil in two concentrations in different solvents.

Table II.

Solvent	Concentration g./l.	$E_{328\,m\mu}$	Correction for solvent	Corrected $E_{328\,m\mu}$	$E^{10\,g./l.}_{328\,m\mu}$
Chloroform	13·0	0·92	0·04	0·88	0·68
,,	6·4	0·47	0·04	0·43	0·67
Alcohol	13·0	0·96	0·09	0·87	0·67
,,	6·4	0·52	0·09	0·43	0·67
Toluene	13·0	0·90	0·07	0·83	0·64
,,	6·4	0·50	0·07	0·43	0·67

It will be seen that the various conditions give good agreement for $E^{10\,g./l.}_{328\,m\mu}$.

Vitameter values as compared with spectrographic determinations.

It is of course vital to the value of the readings in the vitameter that they agree with or stand in some constant relation to values determined spectrographically. We have carried out numerous determinations on cod-liver oils, halibut-liver oils and also various concentrates that give good agreement, and the values give the usual parallelism with the blue values obtained.

Table III shows results which have been found after the present technique had been worked out.

Table III.

Sample	Colour 2 cm. layer		Free fatty acid g./100g.	Kreis test R.V.	B.V. calculated for 0·04 g. oil	$E^{10g/l.}_{328\,m\mu}$	
	Y.V.	R.V.				Spectrograph	Vitameter
Cod-liver oil 1	1·5	0·0	0·95	3·0	7·7	0·50	0·46
Concentrate of same	—	—	—	—	15·5	0·42	0·39
,, (quick method)	—	—	—	—	—	0·42	0·38
Cod-liver oil 2	4·4	0·4	1·40	6·0	17·0	0·89	0·90
Concentrate of same	—	—	—	—	29·0	—	0·77
,, (quick method)	—	—	—	—	—	—	0·77
Cod-liver oil 3	7·0	1·2	3·20	9·6	15·0	0·76	0·75
Concentrate of same	—	—	—	—	—	0·68	0·62
,, (quick method)	—	—	—	—	—	0·68	0·66
Cod-liver oil 4	3·0	0·2	1·00	15·0	19·0	0·97	1·03
Cod-liver oil 5	2·0	0·0	0·80	3·0	18·0	0·94	0·92
Halibut-liver oil 1	1040	205	27·50	*ca.* 600	1600	47	49
Halibut-liver oil 2	35	5·6	6·00	*ca.* 100	1750	50	49
Herring oil concentrate	—	—	—	—	0·9	0·073	0·066
Ether extract of vitamin preparation (thick syrup; ether-soluble matter = 1·2 g./100 g., sp. gr. = 1·32)			Sample	3	3·3	0·124	0·117
			,,	24	15·0	0·38	0·39
			,,	73	10·5	0·25	0·23

The blue value, colour and Kreis test have all been determined in a standard Lovibond Tintometer. The last test has been carried out with 5 ml. cod-liver oil + 5 ml. concentrated hydrochloric acid + phloroglucinol and measured in a 2 cm. layer [Taffel and Revis, 1931; Notevarp and Pillgram-Larsen, 1932].

Practical applications of the vitameter.

$E_{328\,m\mu}$ *of oils and their concentrates.* The directions for the determination of vitamin A by means of the absorption at 328 $m\mu$ regard it as necessary to carry out the determination on the unsaponifiable fraction, except in the case of very potent preparations. It seems, though, as if the relation between the biological potency and spectrographic value is chiefly based on E of cod-liver oils themselves [Coward *et al.*, 1932; Chevallier and Chabre, 1933]. Separation of the unsaponifiable fraction is a considerable complication and greatly increases the demand for accuracy if the results are to be reliable. By the introduction of the photographically recording vitameter the measurement of absorption has become quite a simple operation with few sources of error, and if it were possible to avoid the preparation of the concentrate the determination of E would approach the determination of the blue value in simplicity. This latter is now in very extensive use for commercial and other mass analysis, but it is known to be a less good measure of the vitamin content than the absorption; it is messy and uses an unstable reagent, and it embraces a considerable number of sources of error. It would seem a great improvement if it were possible to replace it by the vitameter. We have therefore carried out a number of determinations on oils and their concentrates to obtain an estimate of the intrinsic absorption of the oils. These values will be found in Tables III and IV. Table III gives the values for three oils determined both spectrographically and in the vitameter, the values in Table IV are from the vitameter alone.

All due care has been taken when preparing the concentrates, the values marked * refer to concentrates prepared by a rapid method we have developed, which will not be described in detail here. The principle is that only a small amount of oil is saponified and the soap solution extracted with a known volume of toluene. This is then washed, dried and used directly for the determination. All values have been corrected for the absorption of the solvent. The values for the concentrates are calculated so that they correspond with the amount of oil from which they have been prepared as the amount of concentrate is of no interest in this connection.

Table IV.

Sample	Quality	Colour 2 cm. layer		Free fatty acid g./100 g.	Kreis test R.V.	B.V. calculated for 0·04 g. oil	$E^{10g/l}_{328m\mu}$		$\frac{E \text{ conc.}}{E \text{ oil}}$
		Y.V.	R.V.				Oil	Concentrate	
L. 14	Light medicinal	1·5	—	0·35	2·3	10·0	0·58	0·51*	0·88
L. 15	,,	1·5	—	0·45	2·0	12·0	0·65	0·62*	0·95
L. 28	,,	1·5	—	0·25	4·6	11·0	0·69	0·65*	0·94
567	,,	1·4	—	0·25	*ca.* 5·0	8·8	0·52	0·44	0·85
								0·47*	0·90
559	,,	1·7	—	0·35	5·0	8·0	0·50	0·44	0·88
A.J. 3	,,	1·5	0·0	0·95	3·0	7·7	0·46	0·39	0·85
								0·38*	0·83
P. 215 I	Dark medicinal	4·4	0·4	1·40	6·0	17·0	0·90	0·77	0·86
								0·77*	0·86
P. 132	Fresh	—	—	—	—	—	0·82	—	0·85
	Rancid	2·0	0·0	0·25	14·0	16·4	0·97	0·70*	0·72
272	Crude	7·0	1·2	3·20	9·6	15·0	0·75	0·66*	0·88
								0·62	0·83
P. 182	Poultry brown	30	3·0	0·50	31·5	8·5	0·73	0·42*	0·58
P. 323	Industrial	100	15	32·0	9·0	6·5	0·65	0·33*	0·51

It will be seen that the absorption values of concentrates of medicinal cod-liver oils of high quality are from 83 to 95 % of the corresponding absorption of the oil. Even crude oils with 3·2 g./100 g. free fatty acid have given absorptions in the concentrate of 83 and 88 % of that of the oil. To determine the $E_{328m\mu}$ of pure medicinal oils it would therefore appear unnecessary to separate the unsaponifiable fraction, and in the case of cruder oils it seems only necessary when a high degree of accuracy is needed. The intrinsic absorption of these oils must be small, especially as it cannot *a priori* be supposed that the concentrates can be prepared entirely without loss.

In this connection it is interesting to note that Morgan *et al.* [1934] state that the biological activity of concentrates is 85 to 95 % of the corresponding value of the oils and that Evers and Smith [1934] find values of E for concentrates of fresh medicinal cod-liver oils which are 83–94 % of the values for the oils themselves.

E *determined in the vitameter in relation to the blue value.*

We have obtained hundreds of parallel determinations of E and the blue value, as these are carried out simultaneously as a rule, and have found a satisfactory proportionality between the two. We are aware that this is not usually found for the oils by other authors [Coward *et al.*, 1932, *etc.*], whereas it is found for the concentrates [*e.g.* McWalter, 1934]. Our evaluation of the blue value differs from the usual, however, so it is necessary to explain this.

It is a well-known fact that the number of Lovibond glasses necessary for matching the antimony trichloride blue value is not proportional to the concentration of chromogen. If it is not possible to obtain a direct match for 0·04 g., owing to the depth of the colour, I have found that the true blue value, *i.e.* the value that would have been found if the matching of 0·04 g. in a 1 cm. cell had been possible, can be calculated by a formula of the following type:

$$B = b . d^x,$$

where b is the blue value actually measured, and d the dilution factor. x was originally determined to be 0·7 from a very great number of blue values carried out at different concentrations [Notevarp and Hjorth-Hansen, 1932]. Later on it was possible to check the formula over a much greater range (B.V. = 4 to B.V. = 150) in relation to spectrographic measurements, and it was found that $x = 0·8$ gave the best agreement [Notevarp and Weedon, 1933]. Detailed publications dealing with the subject are in preparation.

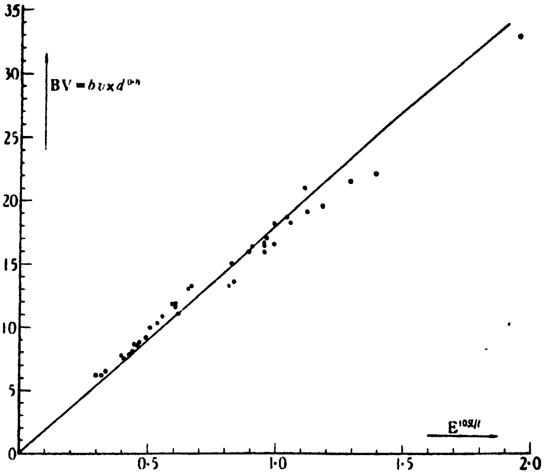


Fig. 3.

Fig. 3 shows how blue values calculated in this way give proportionality with E determined in the vitameter.

Destruction of the vitamin. Differential determinations.

The intrinsic absorption of absorbing oils may be eliminated by separating the unsaponifiable fraction, but this is of course not possible when the absorbing substances are of the same chemical nature as the vitamin. This will often be the case with oils and fats of a low vitamin potency, such as herring oils, muscle oils of other fat fishes, vitaminised margarine, butter *etc.*

To estimate the absorption due to vitamin A it will then be necessary to remove either the foreign absorbents or the vitamin. The removal of pigments by means of adsorbent carbon is mentioned by Gillam *et al.* [1933].

It has been attempted to remove the vitamin in various ways. McWalter [1934] describes the effect of heat and aëration; irradiation has also been mentioned. We have tried these various methods of destruction, with the result that oxidation and heat have been rejected as uncertain. Oxidation will often give rise to substances that absorb light at 328 $m\mu$, especially when there are minute quantities of fat present. Irradiation seems to give much better results; even when an oil is dissolved direct in alcohol, it appears to be possible to destroy vitamin A by light without the formation of appreciable amounts of absorbing substances. The difference agrees with what would be expected from the absorption of the concentrate or the blue value.

This subject will also be treated more in detail in a later publication, as there are still points that need closer investigation. Thus we have observed a hitherto inexplicable rise in the absorption of concentrates after destruction in a few cases. I will therefore only mention a few differential determinations here. The destruction has been effected through irradiation with a 500 watt Osram Vitalux lamp, the solutions being placed about 4 cm. from the surface of the lamp, usually for about 6 hours. The samples were cooled by a strong current of air, yet the temperature of the solution rose to about 40°.

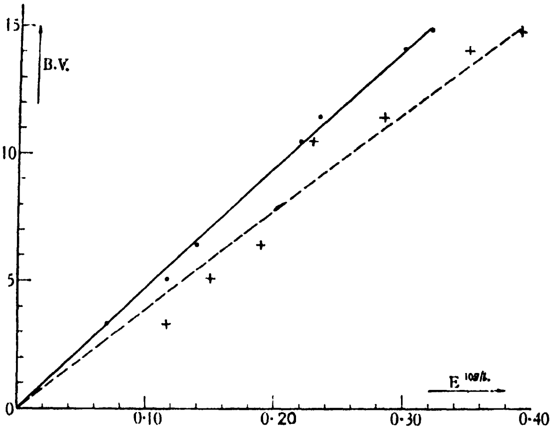


Fig. 4. + Gross values. • Net values (differential).

Fig. 4 shows the results for the ether extracts of samples of a vitamin preparation intended as a substitute for cod-liver oil. The gross absorption values do not agree very well with the blue values, while the differential determinations show as good agreement as may be expected from these methods. The blue values and E have been determined absolutely independently.

I would further mention that concentrates of a pure light cod-liver oil, or a pure halibut-liver oil, give values after irradiation from 5 % of the original value down to 0. This would appear to indicate that no substances that absorb light at 328 $m\mu$ are produced during the irradiation.

Destruction of the vitamin in the undiluted oil.

A sample of cod-liver oil was left on the roof for about 6 months (April 4th to October 10th, 1934) in a full, stoppered bottle of white glass. The following results were found:

	Colour 2 cm. Y.V.	Free fatty acid g./100 g.	Kreis value R.V.	Peroxide value	B.V. 0·04 g.	$E^{10\text{g}/\text{l}}_{328\,m\mu}$
Oil before irradiation	2·6	0·85	10·5	5·5	9·0	0·52
Oil after irradiation	0·3	1·00	1·4	6·3	0·5	0·027

The irradiated oil has only about 5 % of the original E value, which agrees with the blue value. This may indicate that the light has destroyed the vitamin without forming new absorbing substances, but the possibility exists that the irradiation has destroyed other absorbents.

E *for the unsaponifiable fraction of herring oil, mackerel oil and margarines.*

As a last instance of determinations of E by means of the vitameter I will mention some values for concentrates of herring and mackerel oils and margarines, in Table V. The values are calculated so as to correspond with the amount of original substance.

Table V. *Concentrates of different oils and margarines.*

Sample		$E_{328\,m\mu}$ calc. for 10g. oil/l.			Blue value calc. for 0·04 g. oil ($B=b.d^{0\cdot8}$)
		Gross value (initial)	After destruction	Net value difference	
Herring oils:					
From fresh herrings	I	0·081	0·014	0·067	2·4
	II	0·119	0·059	0·060	2·4
Salted	Males	0·072	0·031	0·041	1·6
	Females	0·057	0·018	0·039	1·8
Herring meal		0·066	0·029	0·035	0·9
Mackerel oils:					
From frozen mackerel	I	0·173	0·072	0·101	4·0
	II	0·157	0·030	0·127	5·0
Margarines:					
Vitaminised I		0·019	0·012	0·007	0·3
II		0·013	0·009	0·004	0·15

The unsaponifiable fractions have been prepared in the usual way or by the rapid method mentioned earlier. E and the blue value have been determined in the concentrate, and then the solution has been irradiated until the E value was constant.

SUMMARY.

The Hilger vitameter A has been equipped with a simple device for photographic recording of the readings, which makes it possible to determine $E_{328\,m\mu}$ of oils, concentrates and the like with considerable accuracy.

The vitameter values give good agreement with spectrographic values, both for oils and their concentrates. Determinations have been carried out on pure and crude cod-liver oils, halibut-liver oils, concentrates of herring oil and extracts of commercial vitamin A preparations.

The vitameter values agree with the blue values found. The absorption of the unsaponifiable matter of pure cod-liver oils approaches that of the oil itself; normally 85–90 % is found. The reduction in absorption may be chiefly due to unavoidable losses during the preparation of the concentrate.

Vitamin A may be destroyed by irradiation, and it is thus possible to determine the vitamin by the difference in absorption before and after exposure to strong light. This eliminates any absorption due to substances other than vitamin A.

The Hilger vitameter A equipped with a device for photographic recording has proved to be a very useful instrument for the rapid determination of $E_{328\,m\mu}$ as a measure of vitamin A, and may be expected in many cases to replace the tintometer for rapid assay of the vitamin.

The author wishes to express his sincerest thanks to H. W. Weedon for much valuable help, especially in carrying out the spectrographic determinations and translating the manuscript. Further to L. Aure, who has carried out the majority of the vitameter determinations and a considerable part of the analytical work. Several other members of the staff have also contributed interested and helpful work.

Our thanks are due to Prof. B. Helland-Hansen, of the Geophysical Institute, Bergen, for placing their spectrograph at our disposal.

REFERENCES.

Chevallier and Chabre (1933). *Biochem. J.* **27**, 298.
Coward, Dyer and Morton (1932). *Biochem. J.* **26**, 1593.
Evers and Smith (1934). *Quart. J. Pharm.* **7**, 476.
Gillam, Heilbron and Morton (1933). *Biochem. J.* **27**, 878.
League of Nations Commission 1934. *Z. Vitaminforsch.* **3**, 279 (1934).
McWalter (1934). *Biochem. J.* **28**, 472.
Morgan, Edisbury and Morton (1934). *Chem. Ind.* **53**, 1083.
Notevarp and Hjorth-Hansen (1932). Årsberetning vedkommende Norges Fiskerier, 1931, No. 6.
—— and Pillgram-Larsen (1932). Årsberetning vedkommende Norges Fiskerier, 1931, No. 5, p. 31.
—— and Weedon (1933). Årsberetning vedkommende Norges Fiskerier, 1932, No. 3, p. 51.
Taffel and Revis (1931). *J. Soc. Chem. Ind.* **50**, 87 T.

CL. MONOLAYERS OF PROTEOLYTIC ENZYMES AND PROTEINS.

III. ENZYME REACTIONS AND PENETRATION OF PROTEIN MONOLAYERS.

BY JACK HENRY SCHULMAN AND
ARTHUR HENRY HUGHES[1].

From the Laboratory of Colloid Science, Cambridge.

(*Received March 23rd, 1935.*)

IN a recent publication Schulman and Rideal [1933] have studied the action of pancreatin and of specific proteolytic ferments on monolayers of protein. The changes which occurred in these films were such as might be expected from the properties of enzymes in bulk. Pancreatin differed from the specific proteolytic enzymes examined in that inactivation for reaction in the bulk phase did not destroy its surface reactivity on protein monolayers.

It was at that time suggested that this behaviour might be ascribed to a free enzyme or active group which on separation from some colloidal carrier could only operate on a substrate presented in the form of a monolayer. This original hypothesis has now been modified. In respect to crude enzyme preparations the situation is rendered more complex by the presence of highly capillary active materials of the class of the long chain fatty acids bound in such a way that they cannot be removed by fat solvents such as chloroform, light petroleum or benzene. It was found, however, that they could be removed by hot ethyl alcohol.

Further work showed that the surface activity of these inactivated pancreatin solutions was due to a fatty acid-protein complex. This reacts in a similar manner to pancreatin itself in respect to the p_H optimum and inhibition by sodium fluoride. It further has the property of replacing a film of tripalmitin by a film indistinguishable from one of palmitic acid.

Purified enzyme preparations such as Northrop's proteinase and crystalline pepsin and proteinases prepared by the method of Waldschmidt-Leitz caused hydrolysis of protein monolayers as they would in the bulk phase, and these preparations were found to contain no alcohol-soluble material. Before proceeding further in the study of these surface reactions it was thought advisable to examine the mechanism by which a film of one substance may be displaced by another substance present in solution, in order to differentiate the two phenomena as clearly as possible. The displacement mechanism as distinct from a chemical reaction is of intrinsic importance and presents some novel features. Experiments will be described showing that two processes may take place; first penetration of the original film by the second substance to form a stable mixed film, or alternatively complete displacement of the one by the other may ensue. The result is in some measure specific to the type of molecules engaged.

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[1] Beit Memorial Research Fellow.

lowering. The tripalmitin film on the other hand exerts too high a maximum surface pressure to be displaced.

It is of interest that if we study displacements by a soap-protein complex instead of by soap alone, no displacements occur in solutions more acid than about p_H 3–4, since there is no free fatty acid, while again beyond p_H 12 no displacement occurs, either of tripalmitin or of protein films. The protein, by forming this relatively stable complex in acid and alkaline solutions, thus greatly reduces the concentration of free fatty acid or soap and accentuates the apparent p_H optimum of film displacements.

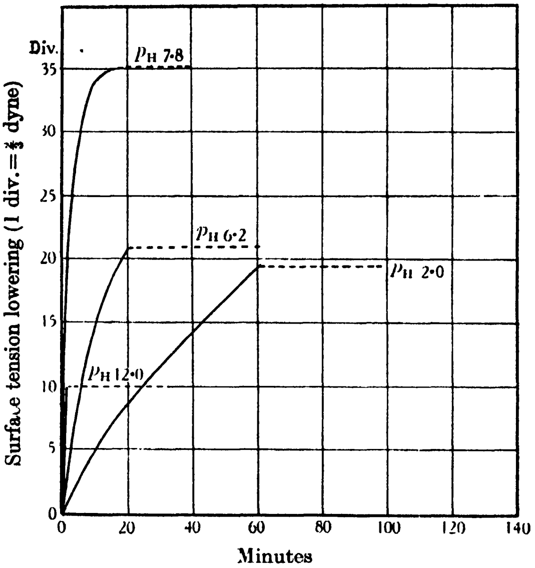

Fig. 4. Temperature 18–20°; Na oleate 0·0005 %.

The effect of 1 % sodium fluoride in coagulating a caseinogen-sodium oleat complex has been referred to. Inhibition of displacements results owing to th diminished concentration of free soap. Sodium fluoride has no effect on di placement caused by soap in the absence of protein.

Belfanti [1924] has described the isolation of fatty substances (Y) fron pancreatin by alcoholic extraction. Treatment of the alcoholic extract witl ether effected a further separation into a water-soluble and a water-insolubl fraction. Belfanti found that the former substance was strongly haemolyti and the latter much less so. These findings have been re-examined in the cours of the present work. Commercial pancreatin was extracted with alcohol sever times in the cold. The residue was found to be non-haemolytic while the origina pancreatin was strongly haemolytic. Further, the whole of the haemolyt principle was concentrated in the alcoholic extract. This extract on evaporati was indeed insoluble in water but dissolved readily in alkali. It behaved in f like a complex mixture of fatty acids. The haemolytic properties are, theref not surprising since sodium oleate at a concentration of 0·25 mg./ml. ca instantaneous haemolysis of washed guinea-pig erythrocytes at room tempera Again the precipitate obtained on acidifying alkali-treated pancreatin lik after redissolving in a neutral solution contained all the haemolytic power original pancreatin. It is significant further that the following specific proteinases obtained from pancreatin cause no haemolysis of guinea-pig erythrocytes: (1) Pancreatin freed from lipase and lipoids by alumina *B* adsorption. (2) Northrop's crystalline trypsin. (3) Northrop's chymotrypsin.

It would appear that haemolysis is attributable to the cholesterol-lecithin portion of the erythrocyte wall being exposed to the peptising action of the protein-fatty acid complexes present in ordinary pancreatin and not to proteolysis of any protein in the membrane.

Langenbeck [1934] describes several organic catalysts of the type of benzyl alcohol which in concentrations of some 20 mg./ml. cause a sevenfold acceleration of the autolysis of certain fats and esters. It is perhaps significant that sodium oleate acts in a manner similar to these compounds of Langenbeck under the same conditions, *i.e.* at 80–90° and at much smaller concentrations.

For example 6 ml. of a solution of a protein-fatty acid complex containing 15 mg. of solid matter obtained from pancreatin treated with NaOH increased the autolysis of 0·1 ml. of monobutyrin more than seven times at a temperature of 85°. In 3½ hours 6·75 ml. *N*/80 NaOH were required for the titre at 85° compared with 0·9 ml. in the absence of the peptising agent. With 10 mg. sodium oleate in 3 hours at 95° 5·8 ml. *N*/80 NaOH were required compared with 1·8 ml. *N*/80 NaOH when the sodium oleate was absent. It is clear that sodium oleate increases the autolysis in a quite definite manner but the increased autolysis of the protein-soap complex is rather marked. It was further noticed that on addition of sufficient ethyl alcohol to render homogeneous an emulsion of 0·1 ml. monobutyrin in 5 ml. H_2O, the rate of autolysis was increased compared with that of an alcohol-free emulsion, while addition of soap no longer caused increased autolysis.

Increased autolysis due simply to increased dispersion of an insoluble oil by a suitable emulsifying agent would seem, therefore, to be a factor which cannot be neglected in these systems.

SUMMARY.

It has been found possible to extract from pancreatin a fatty acid-protein complex. This complex simulates the action of ferments on monolayers of protein and fats spread at an air-liquid interface. This behaviour explained the activity towards surface films of pancreatin preparations which were inactive in bulk. Specific proteolytic ferments do not contain this complex and consequently behave towards protein monolayers as they do in bulk.

We are much indebted both to Prof. Northrop for specimens of the purified proteolytic ferments and to Dr D. R. P. Murray for helpful criticism.

REFERENCES.

Belfanti (1924). *Biochem. Z.* **154**, 148.
Langenbeck (1934). *Ber. deutsch. chem. Ges.* **67**, 387.
du Noüy (1926). Surface equilibria of colloids, p. 160.
Schulman and Rideal (1933). *Biochem. J.* **27**, 1581.

CLI. MONOLAYERS OF PROTEOLYTIC ENZYMES AND PROTEINS.

IV. MIXED UNIMOLECULAR FILMS.

By JACK HENRY SCHULMAN and
ARTHUR HENRY HUGHES[1].

From the Laboratory of Colloid Science, Cambridge.

(*Received March 23rd, 1935.*)

WHILST a considerable knowledge exists as to the properties and reactions of unimolecular films of pure substances at an air/liquid interface, comparatively little is known of the behaviour of two or more different substances present together as a mixed film. Such information is of importance in connection with reaction kinetics in unimolecular films and also in regard to the properties of naturally occurring membranes, which cannot be treated in terms of one substance or even of one class of substance.

The most important observations on mixed films are those of Adam [1928] on the so-called "condensing" effect of large molecules on smaller molecules when both are present in a mixed unimolecular film. Thus addition of cholesterol or of tripalmitin to myristic acid, which itself forms an expanded type of film with a limiting area at zero compression of about 50 sq. Å., causes the myristic acid to assume the liquid condensed state with a much smaller limiting area per molecule. According to Adam the vibratory movements of the hydrocarbon chains, which are of large amplitude in the expanded state, are greatly hindered by the large and physically inert cholesterol or tripalmitin molecules. Adam further emphasised that there was no evidence of direct compound formation in the type of mixed films examined by him, since the two-dimensional vapour-pressure is not reduced more than would be expected from the proportion of cholesterol molecules present in the film. In a less simple case Leathes [1925] has observed a similar effect of cholesterol in condensing an expanded film of lecithin, a point of some interest in connection with the stability of living cell membranes. By the method of surface potentials Hughes [1935] showed that the condensation in this case was not accompanied by any appreciable change in the dipole moments of the two components, but that the molecular orientation was retained to a higher compression in an equimolecular mixture than in any other mixture.

In the present work a further study has been made of more varied types of mixed films and it is shown that the condensing effect is but one of several distinct phenomena observable in these systems. Thus it was found that the stability of mixed films was closely related to an effect sometimes encountered in studying chemical reactions in unimolecular films. This effect, which may be termed "film penetration", is met with in certain cases when a capillary active substance (B) is introduced into the solution beneath a pre-existent film of another substance (A). There are three possibilities. First, the film A may be completely replaced by a unimolecular film of B; secondly, B may penetrate the film of A and produce a stable mixed film of A and B; or finally neither replacement nor penetration occurs, *i.e.* no change can be detected in the original film. Examples of each behaviour have been observed and the relation of each to the physical and chemical properties of the system is discussed.

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[1] Beit Memorial Research Fellow.

EXPERIMENTAL.

Equimolecular mixtures were prepared in light petroleum solution of the following pairs of substances: oleic acid and hexadecyl alcohol; sphingosine and hexadecyl alcohol; oleic acid and tripalmitin; and mixtures in the molecular proportions of 3 : 1 respectively were prepared of oleic acid and triolein; oleic acid and tripalmitin; hexadecyl alcohol and triolein.

The mixed films obtained by spreading from these solutions were examined by the methods of surface potentials and of surface pressures. The dark-field microscope technique of Zocher and Stiebel [1930] was applied to examine cases of doubtful homogeneity of the surface film. The results for the surface pressures and surface potentials are shown in the accompanying figures, where n as abscissa represents the total number of molecules per square centimetre of both kinds.

(i) *Oleic acid and tripalmitin* (Figs. 1 and 2).

At p_H 7·4 ($M/25$ phosphate buffer) oleic acid forms a liquid expanded film of the soap with a limiting area per molecule at zero compression of 53 sq. Å. At the same p_H the mixture gives a limiting area of 55 sq. Å. per molecule and on compression the surface pressure and potential rise uniformly till $n = 3{\cdot}1 \times 10^{14}$ mols./sq. cm. Beyond this point the surface pressure remains constant at 21–22 dynes/cm., while the surface potential falls rapidly to a constant value of 175 mv. at $n = 5{\cdot}35 \times 10^{14}$. The surface pressure level corresponds to the equilibrium breaking pressure of a tripalmitin film and up to this point the observed surface potential curve is that corresponding to a simple mixed film of the two constituents. On further compression it must be supposed that the tripalmitin is collapsing under the pressure of the oleate, which remains as a unimolecular film. The final surface potential is the maximum obtainable with sodium oleate alone at this p_H. Optical examination of the mixed film reveals numerous minute crystallites in the broken film. These are not observed with a pure tripalmitin film, which shows long parallel strain lines on fracture and has no tendency to break up into these highly characteristic crystallites noticed in the mixed film. In other words the oleic acid may be said to "peptise" the tripalmitin.

The condensing action of tripalmitin on the oleate molecules is not great in the 1 : 3 ratio. The area per molecule at zero compression for the oleate alone is 53 sq. Å. at this p_H of 7·4, while in the mixture it is 50 sq. Å. In the 1 : 1 ratio the area per molecule is reduced to 40 sq. Å. at zero compression, assuming that the tripalmitin molecules have a constant area per molecule of 60 sq. Å.

Compression of the 1 : 1 mixed film gives the same collapse point on the surface pressure curve and a similar peak in the potential curve at a higher surface potential corresponding to the greater number of molecules with the higher electric moment (μ for tripalmitin $= 10{\cdot}0 \times 10^{-19}$ E.S.U. and for oleic acid $2{\cdot}1 \times 10^{-19}$ at p_H 2, $1{\cdot}0 \times 10^{-19}$ at p_H 7·0). On further compression the potential falls to that of sodium oleate alone. The calculation of the condensing effect of the tripalmitin on the oleate molecules is made as follows.

Let n = total number of molecules/sq. cm. of surface. In the 1 : 1 ratio let A be area/mol. in sq. Å. of oleate molecules, assuming tripalmitin to have an area/mol.

of 60 sq. Å. The observed value of n at zero compression of mixed film $= 2{\cdot}0 \times 10^{14}$, $\frac{1}{2}n \times 60 \times 10^{-16} =$ area occupied by tripalmitin in 1 sq. cm. of film, therefore $(1 - 30 \times 10^{-16}\, n) =$ area occupied by oleate, *i.e.*

$$A = \frac{(1 - 30 \times 10^{-16}\, n)}{\frac{n}{2}} \times 10^{16} = 40 \text{ sq. Å.}$$

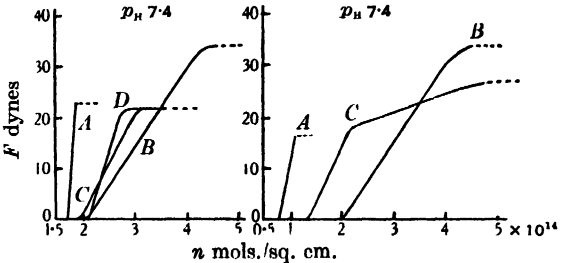


Fig. 1. Fig. 3.

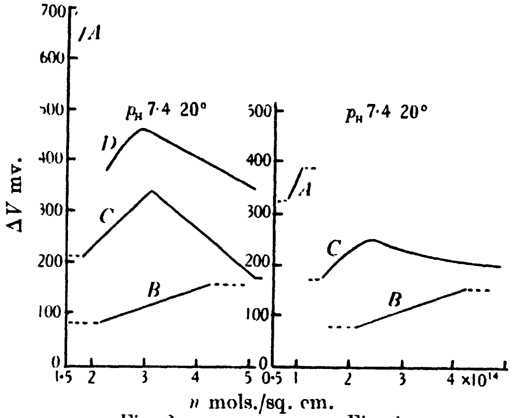


Fig. 2. Fig. 4.

Figs. 1 and 2. *A*, Tripalmitin; *B*, oleic acid; *C*, 1 tripalmitin : 3 oleic acid; *D*, 1 tripalmitin : 1 oleic acid.

Figs. 3 and 4. *A*, Triolein; *B*, oleic acid; *C*, 1 triolein : 3 oleic acid.

(ii) *Oleic acid and triolein* (Figs. 3 and 4).

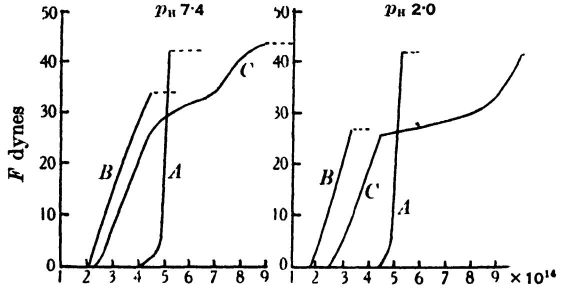
The 3 : 1 mixed film is homogeneous and liquid expanded from zero compression at $n = 1{\cdot}4 \times 10^{14}$ mols./sq. cm. up to $n = 2{\cdot}5 \times 10^{14}$ mols./sq. cm. At higher values of n the surface potential decreases while the surface pressure curve rises much less steeply than over the initial portion of the curve, indicating partial collapse of the triolein. The final collapse pressure and potential correspond to a mixture of triolein and oleic acid richer in oleic acid than the original 3 : 1 oleic acid : triolein.

The mutual effect of the two substances is negligible in that the observed limiting area per molecule in the mixed film (at zero compression) is within experimental error equal to that calculated for the simple mixture.

(iii) *Hexadecyl alcohol and oleic acid* (Figs. 5 and 6, 7 and 8).

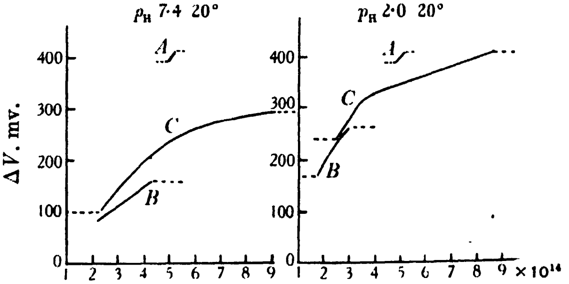
This mixed film was examined at p_H 2 (N/100 HCl) and at p_H 7·4.

At p_H 2 the mixed film collapses at the same pressure as oleic acid alone, indicating that the hexadecyl alcohol is displacing the oleic acid. The surface potential of the mixed film shows that in the early stages of compression a uniform mixed film is obtained, but that ultimately the potential rises to 410 mv. corresponding to a film of hexadecyl alcohol alone. The observed value of n at the final collapse point further shows that about half of the original total number of molecules in the film have been expelled, presumably as minute droplets of oleic acid. The condensing action of hexadecyl alcohol on oleic acid is negligible at this p_H (*cf.* Table I).



n mols./sq. cm.

Fig. 7. Fig. 5.



n mols./sq. cm.

Fig. 8. Fig. 6.

Figs. 5–8. *A*, Hexadecyl alcohol; *B*, oleic acid; *C*, 1 mol. alcohol : 1 mol. acid.

At p_H 7·4 the behaviour is different in that the surface potential never rises to that of hexadecyl alcohol alone. The surface pressure does not show a sharp collapse point corresponding to incipient collapse of the oleic acid but continues rising though less steeply beyond this point—as in the case of triolein and oleic acid. This difference in behaviour must be attributed to the formation of a relatively stable complex between the ionised oleate molecule and the hexadecyl alcohol, which increases their mutual adhesion at p_H 7·4 but does not exist at p_H 2.

Again, the limiting area at zero compression in the mixed film at p_H 7·4 is greater than that calculated assuming the areas of the components to be unchanged. Thus if we assume the hexadecyl alcohol to be unaltered, with an area per molecule of 20 sq. Å. the calculated area per molecule of oleic acid in the mixed film is 75 sq. Å. instead of 53 sq. Å. when alone. This must mean an expansion of the hexadecyl alcohol by the oleic acid molecules at this p_H (*cf.* Table I).

It is possible to calculate the surface potential in the mixed film from the known electric moments (μ_1, μ_2) of the two constituents, assuming that these have not been altered in the mixed film from the original values. We may write $\Delta V = 4\pi \frac{n}{2}(\mu_1+\mu_2)\times 300\times 10^3$ mv. for the surface potential of the mixed film of two components in equimolecular properties, n being the total number of molecules per sq. cm.

At p_H 2 it so happens that the vertical components of the electric moment per molecule of oleic acid and hexadecyl alcohol are identical ($\mu = 2{\cdot}1 \times 10^{-19}$ E.S.U.); and as seen in Fig. 6 the $\Delta V/n$ curve for the mixture is an extension of the $\Delta V/n$ curve for oleic acid or hexadecyl alcohol alone. At p_H 7·4 however, the value of μ for oleic acid has fallen to $1{\cdot}0 \times 10^{-19}$ E.S.U., owing to ionisation, and the mixed film of oleate and alcohol has a surface potential close to the value calculated by the above equation. At $n = 4{\cdot}0 \times 10^{-14}$ mols./sq. cm. (25 sq. Å.) the observed value of ΔV is 200 mv. The calculated value is 230 mv.

(iv) *Sphingosine and hexadecyl alcohol* (Figs. 9, 10, 11 and 12).

A much clearer example of complex formation in mixed films is afforded by an equimolecular mixture of sphingosine and hexadecyl alcohol.

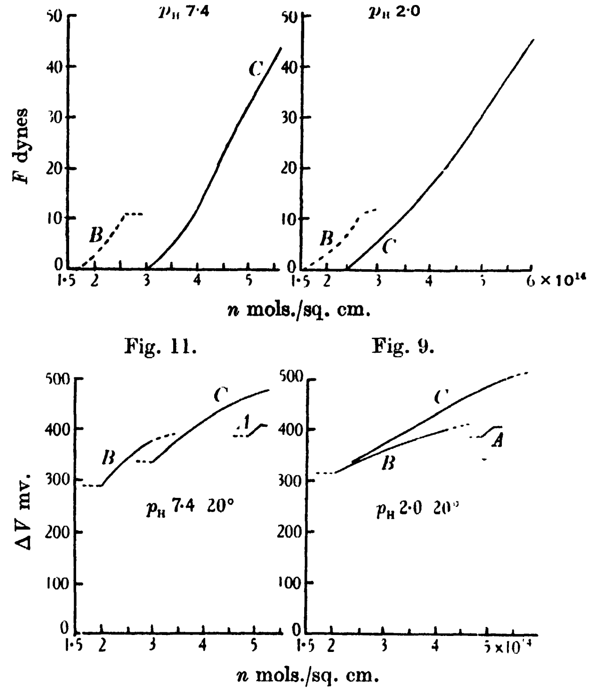


Fig. 11. Fig. 9.

Fig. 12. Fig. 10.

Figs. 9–12. *A*, Hexadecyl alcohol; *B*, sphingosine; *C*, 1 hex. alc. : 1 sphingosine.

Sphingosine alone on acid or neutral solutions forms unimolecular films which slowly dissolve in the underlying solution. The curves *B* for the surface pressures and potentials of sphingosine alone at p_H 2 and p_H 7·4 are obtained by compressing the films as rapidly as possible; the films are liquid expanded but the recorded areas per molecule are probably too small on account of the solubility effect. If now the equimolecular sphingosine-hexadecyl alcohol mixture be examined a stable mixed film is found at p_H 2 or p_H 7·4. This film collapses at an area per molecule of 20 sq. Å. and has a surface potential much higher than that of hexadecyl alcohol. The sphingosine is clearly not displaced by the hexadecyl alcohol although the latter can exert a much higher two-dimensional surface pressure than sphingosine. The alcohol must be supposed to hold the sphingosine in the film in an oriented mixed monolayer, or in other words the solubility of the sphingosine film is greatly reduced by the presence of the hexadecyl alcohol. The rise of surface potential is due to the high vertical component of the electric moment of sphingosine. As in the case of oleic acid and hexadecyl alcohol we may calculate the surface potential in the mixed film. The value of μ for sphingosine at p_H 7·4 is $3{\cdot}5 \times 10^{-19}$ E.S.U. at $n = 3{\cdot}0 \times 10^{14}$ mols./sq. cm., and for hexadecyl alcohol $\mu = 2{\cdot}1 \times 10^{-19}$ E.S.U. ($n = 5{\cdot}0 \times 10^{14}$ mols./sq. cm.).

The calculated value of ΔV in the mixed film at 25 sq. Å. ($n = 4{\cdot}0 \times 10^{14}$) is 422 mv. The observed value is 420 mv. Compression to $n = 5 \times 10^{14}$ (20 sq. Å.) gives an increase of ΔV to 470 mv.; the calculated value is 518, but the discrepancy is ascribable to partial collapse of the mixed film. It is clear that the potential of a mixed film may be equal to, less than or greater than that of one component, depending on the actual dipole moment of the other component and the degree to which the orientation is retained on compression of the mixture.

The condensing effect.

In the case of the hexadecyl alcohol and oleic acid mixed film at p_H 7·4 it was noted that the oleic acid apparently causes an expansion of the hexadecyl alcohol film, and that the hexadecyl alcohol is thus not condensing the expanded film of oleic acid. It is useful to summarise these area changes in the following manner. If there is no mutual effect on the limiting area per molecule, and x is the mol-fraction in the mixture of component 1, we may write:

$$A = A_1 x + A_2 (1 - x),$$

where A is the calculated area per molecule at zero compression, A_1 and A_2 being the values for the two components.

In Table I these calculated values are compared with the observed values in the various mixed films.

Table I.

Components		(Sq. Å.)						
1	2	p_H	x	A_1	A_2	A (calc.)	A (obs.)	Difference
Hexadecyl alcohol	Oleic acid	2	0·5	21	55	38	41	+3
		7·4	0·5	21	53	37	48	+11
Hexadecyl alcohol	Sphingosine	2	0·5	21	(67)	(44)	42	(−2)
		7·4	0·5	21	63	42	33	−9
Tripalmitin	Oleic acid	7·4	0·25	60	53	55	53	−2
		7·4	0·5	60	53	56·5	50	−6·5
Triolein	Oleic acid	7·4	0·25	132	53	73	72	−1
Triolein	Hexadecyl alcohol	2	0·25	132	21	49	57	+8

(The solubility of sphingosine at p_H 2 makes the estimated area per molecule in this case low.)

The cases of condensation, *i.e.* a negative result in the final column, are seen to occur with sphingosine and hexadecyl alcohol and with the equimolecular mixture of tripalmitin and oleic acid. In each case the expanded film must be considerably reduced in its limiting area. But this effect is clearly not dependent merely on the physical nature of the films of the two components. If instead of sphingosine and hexadecyl alcohol we take oleic acid and hexadecyl alcohol we now observe an increase in total area per molecule, slight at p_H 2 but at p_H 7·4 amounting to a 30 % increase. This must mean that the expanded film of oleic acid is causing an expansion of the condensed film of hexadecyl alcohol.

A similar expansion is noticed in the mixed film of 3 mols. oleic acid to 1 mol. of hexadecyl alcohol, to the extent of a 16 % increase on the estimated value. The original view of Adam that the inert molecules simply hinder the vibrations of the expanded molecules must now be extended to cases such as these where expanded molecules may increase the thermal agitation of the condensed molecules. It is of interest that where both components are in the expanded state, as in the case of triolein and oleic acid, the mutual effect is negligible.

The case of tripalmitin and oleic acid appears to be of the type discussed by Adam, the effect being one of condensation and proportional to the amount of tripalmitin in the mixture. The diversity of the other cases indicates that the area changes in a mixed film may be to some extent specific to the chemical nature of the molecules concerned, in that a complex film may be produced whose characteristics are very different from those of either component.

FILM PENETRATION.

In the introduction mention has been made of the possible effects of introducing a capillary active substance (*B*) beneath a unimolecular film of a substance *A*. Three possibilities were noted, (i) displacement of *A* by *B*, (ii) penetration of *A* by *B* to give a mixed film of *A* and *B*, (iii) no change in the film of *A*.

Examples of the third and simplest case are found when *B* is a relatively soluble substance. Thus ethyl alcohol, ethylamine, benzenesulphonic acid or aniline hydrochloride produces no change in any unimolecular films of insoluble substances so far studied. Again, sodium acetate in concentrations up to 30 % causes no displacement of a unimolecular film of tripalmitin, but sodium oleate at a concentration of 0·0005 % completely displaces the tripalmitin film in 2 hours at p_H 7·4. This same concentration of sodium oleate will not displace a film of hexadecyl alcohol, but penetrates to form a mixed unimolecular film of oleate and hexadecyl alcohol in about 20 minutes. These preliminary results were extended.

The experimental procedure was to inject the constituent *B* beneath the film of *A* spread on a solution of appropriate p_H. The bulk concentration of *B* was 5.10^{-14} %, unless otherwise stated; the temperature was 18–20°. The films were examined by the methods of surface potentials and surface pressures and by the ring surface tension apparatus. It was found that the ease of displacement or of penetration of *A* by *B* depended on several factors:

(*a*) the physical nature of the film;

(*b*) the nature of the penetrating molecules *B*;

(*c*) the bulk concentration of *B*;

(*d*) the degree of adsorption of *B* at the interface as dependent on the Gibbs adsorption relation.

These factors are exemplified in the following cases. The cases of displacement, as opposed to penetration alone, are few and will be considered first.

Displacement. A = tripalmitin or triolein; B = sodium oleate.

(*a*) *Physical nature of film.* At p_H 7·4 a tripalmitin film in the solid condensed state with an area of 58 sq. Å. per molecule at 15 dynes/cm., corresponding to close-packed vertical chains, is completely displaced in 2 hours by sodium oleate. A triolein film in the liquid expanded state at 100 sq. Å. per molecule and 14 dynes/cm. is displaced in about 30 minutes. This indicates that close packing of the film reduces the ease of penetration and thus also of displacement.

(*b*) *Nature of molecules of B.* At p_H 12 sodium oleate does not displace tripalmitin. At p_H 2 oleic acid takes from 3 to 4 hours to displace tripalmitin, but around neutrality the rate of displacement is a maximum. The fact that no displacement occurs at high p_H values is probably related to the much smaller surface tension lowering caused by the soap in this region as opposed to the very large lowering brought about in neutral solutions. The diminished rate of displacement in acid solutions is due to the formation of an emulsion of oleic acid and the resulting diminution in molecular concentration.

(*c*) *Bulk concentrations of B.* The rate of displacement of tripalmitin films by sodium oleate at the fixed p_H of 7·4 depends on the bulk concentration of oleate. At 5×10^{-3} % displacement is complete in about 30 minutes, at 5×10^{-4} % in about 2 hours, while at 5×10^{-5} % it is incomplete in 10 hours.

(*d*) *Surface adsorption of B.* As already mentioned, relatively soluble capillary active molecules such as ethyl alcohol even in high concentrations (up to 5 %) cause no displacement; presumably since the adsorption beneath the film is too small. Very long chain substances, stearic and higher fatty acids and their sodium salts are much less effective than sodium oleate (or myristate) since they are too insoluble in the bulk solution to give a high enough concentration in the Gibbs layer.

Penetration (without displacement).

In these cases the experimental observation is that a film *A* at an initial very high surface compression, say 20 dynes/cm., can admit molecules of a second substance *B*, from the underlying solution, although this substance itself may only be capable of exerting a much smaller surface pressure. The surface pressure rises rapidly to the highest value—up to 40 dynes/cm. in some cases—which can be exerted by *A* alone, while the surface potential may rise, fall or remain unchanged.

An example of this behaviour is afforded by the injection of sodium oleate beneath hexadecyl alcohol.

1 mg. of sodium oleate was injected beneath a film of hexadecyl alcohol at a compression of 12 dynes/cm. spread on 300 ml. of *M*/25 phosphate buffer at p_H 6·5. The initial value of the surface potential of the alcohol film, 410 mv., fell in 20 minutes to 290 mv. and reached a final value of 270 mv. The film changed from the viscous liquid condensed state to a very rigid mixed film. The value of the surface potential of the acid soap film on the free surface of the liquid was 132 mv. Simultaneously the surface pressure rose from 12 to 40 dynes/cm. as measured by the ring method and by the Langmuir trough. At p_H 2 no change in ΔV occurs, μ for oleic acid and hexadecyl alcohol being identical ($\mu = 2{\cdot}1 \times 10^{-19}$), although a similar change in surface pressure takes place as at p_H 6·5. A more spectacular case is shown by the injection of 1 mg. of psychosine sulphate beneath a film of hexadecyl alcohol at 10 dynes/cm. compression and a surface potential of 420 mv. at p_H 2. The surface pressure measured by the ring method rose in 30 minutes to a constant maximum value of 33 dynes/cm., while the surface potential increased by 178 mv. to 598 mv.

Similar large rises in surface pressure and film potential are observed with both sphingosine and psychosine in solution beneath films of hexadecyl alcohol, cholesterol and palmitic acid, and to a lesser extent beneath films of triolein, lecithin and tripalmitin.

Another case is that of sodium hexadecyl sulphate in solution beneath hexadecyl alcohol. Using the sulphate at 5×10^{-4} % the surface pressure of the hexadecyl alcohol film increased from 16 to 40 dynes/cm. in 2 minutes at p_H 2. The corresponding change in surface potential was from 426 mv. to 336 mv. in 2 minutes, finally reaching a value of 291 mv. The remarkable feature to be noticed is that the surface pressure at the free surface of this solution of sodium hexadecyl sulphate was very small; between 5 and 6 dynes/cm. Reduction of the bulk concentration to 5×10^{-5} % merely increased the time of reaching the final equilibrium (which was unchanged), while the surface pressure at the free surface was now less than 1 dyne/cm.

The phenomenon is very general. The film (*A*) may be a long chain acid, alcohol, amine, ether, tripalmitin, triolein, lecithin or cholesterol. The substance in solution (*B*) may be a long chain amine, sulphate, sulphonate, psychosine or sphingosine. Mixed films result in all cases except for fatty acids beneath fats and ether, when displacement results.

The points requiring explanation are, first, the mechanism of penetration; secondly, the formation of a stable mixed film, although one constituent may possess a much higher solubility than the other; thirdly, the fact that the rise or fall in surface potential of the original film corresponds to the entrance of many more molecules of the second component than there was initially room for.

In regard to this last point, it is clear from the experiments, already described in the first section, on those systems where the mixed films can be studied, that whether the surface potential rises or falls depends simply on the respective values of the dipole moments of the two components. In no case is there convincing evidence of any interaction of the dipoles consequent on mixed film formation. It is, however, remarkable that in the cases of film penetration the final value obtained for the surface potential of the mixed film is never far removed from the value corresponding to an equimolecular mixture of the two components—although in the penetration cases the constituent in the underlying solution is present in about 100 times the actual amount of the material in the original film.

In illustration we may refer to the cases of psychosine and sphingosine. In the form of its sulphate, in neutral and acid solutions, psychosine is very soluble in water and insoluble in light petroleum or benzene, thus excluding a study of its mixed films obtained by direct spreading. The related compound, sphingosine, is capable of study by spreading from benzene solution, and its mixed films have been described. Thus the equimolecular mixed film of hexadecyl alcohol and sphingosine is remarkably stable in spite of the much greater solubility of sphingosine in water. Injection of 10^{-3} g. of sphingosine beneath a film of hexadecyl alcohol containing 10^{-5} g. gives a rise of surface potential to a value approximately equal to that of the equimolecular mixture of sphingosine and hexadecyl alcohol at its maximum compression. While it is impossible to measure the dipole moment of psychosine owing to its extreme solubility, yet the similarity of its behaviour to that of sphingosine on injection beneath unimolecular films implies that its action is much the same.

In these cases of film penetration with formation of stable mixed films it is difficult to avoid the conclusion that the stability of the resultant film is due to the formation of a complex molecule of the two constituents whose stability is due in part to the hydrocarbon chains, and whose formation is not attended by radical changes in the dipole moments of the polar portions of the molecule.

A tentative generalisation may now be made.

(1) If in a mixed film of A and B, compression results in expulsion of one component (A) from the oriented monolayer, then injection of B beneath a film of A will result in complete displacement of A. That is, the mixed film is unstable and the AB complex, if it exists at all, is unstable.

(2) If the mixed film is stable on compression, penetration alone will occur on injection of B beneath A, and will result in a stable (AB) mixed film.

The mechanism of penetration and of displacement is at present obscure, but it is highly probable that displacement is in the first stages due to the formation of an unstable complex of the two molecules which is itself rapidly displaced by excess of the second component beneath the film. In the case of penetration of a close-packed solid condensed film such as that of tripalmitin by sodium oleate it is possible that the soap molecules reach the surface through minute cracks in the film and then spread over and peptise the tripalmitin film. In cases of penetration of liquid films such a mechanism is improbable, since it is difficult to visualise cracks in a liquid film. A possible explanation may be found along the following lines. One must suppose that the molecules in the film (A) are in equilibrium with a definite, though small, bulk concentration of its molecules. The equilibrium $A_{\text{film}} \rightleftarrows A_{\text{dissolved}}$ will be disturbed on the introduction of a second molecule B, which would alone set up its own equilibrium $B_{\text{film}} \rightleftarrows B_{\text{dissolved}}$. When a molecule of A leaves the surface, either A or B may now return and an interchange of A and B will be effected to an extent dependent on the relative adhesional forces of A to A, A to B, and B to B. If the attractive forces between A and B are greater than the cohesional forces between A and A or B and B a mixed film of an AB complex will result, whose stability is related to the stability of this complex. Such molecular complexes are formed between hexadecyl alcohol (or cholesterol) and oleic acid, hexadecylsulphonic acid, hexadecyl sulphate, psychosine or sphingosine; and their stability is sufficient to prevent ultimate displacement by the more soluble constituent. In other cases, such as triolein and oleic acid, the complex must be regarded as being very loose, and complete displacement ensues.

SUMMARY.

A study of two-component unimolecular films has been made, the components being long chain alcohols, acids, glycerides, sphingosine and psychosine. On compression of a mixed film one of two phenomena was found to occur: one component may displace the other from the surface or both components may remain as a stable mixed film. In the latter case there is some evidence of the existence of a complex between the two components which does not involve measurable dipole interaction. In these cases also it is possible to obtain the same mixed film by injection of the one component into the solution beneath a film of the other component. Otherwise complete displacement of the film results.

Our thanks are due to Prof. E. K. Rideal for helpful encouragement and advice, and to the Medical Research Council for financial assistance to one of us (J. H. S.).

REFERENCES.

Adam (1928). *Proc. Roy. Soc. Lond.* A **120**, 473.
Hughes (1935). *Biochem. J.* **29**, 430.
Leathes (1925). *Lancet*, ii, 853.
Zocher and Stiebel (1930). *Z. physikal. Chem.* A **147**, 421.

OBITUARY NOTICES.

JUAN PEDIGE CHARLES CHANDRASENA.

(1887–1934.)

Juan Pedige Charles Chandrasena was born in Colombo on March 27th, 1887. He received his early education at Ananda College, Colombo, and always continued throughout his life to render valuable service to Buddhist education and religion. After qualifying as a trained teacher he joined the Government Technical School; his aptitude for Chemistry was encouraged by Dr A. F. Joseph, the Professor of Chemistry at the Ceylon Medical College and, after passing the Intermediate Examination in Science, Chandrasena was appointed Demonstrator in Chemistry at the latter institution on January 1st, 1914. The first examinations for the London Pass Degree in Science were instituted in Ceylon a little later and Chandrasena was one of the first to take the B.Sc. degree locally. In 1920 he proceeded to England on a Government Scholarship and studied at the Imperial College of Science for two years under Prof. Thorpe and Dr Ingold. The work done at the Imperial College, mostly on polycyclic structures, was published in the *Journal of the Chemical Society* (1922, **121**, 1306, 1542, 1552; 1925, **127**, 1677), and led to the Ph.D. degree and the D.I.C. On his return to Ceylon Chandrasena became Lecturer in Chemistry at University College, Colombo, and, on the retirement of Prof. N. Rae in 1933, he was appointed to the Chair of Chemistry, being the first Sinhalese Professor. He was also for some time Registrar of Patents.

Chandrasena became a member of the Biochemical Society in 1923.

Chandrasena's main research interest was with the economic and medical plants of Ceylon; he published two papers on the coconut in this *Journal* (1930, **24**, 1493; 1933, **27**, 3) and a note on atisine (*J. Chem. Soc.* 1933, 740). He had completed a monograph on the Medicinal Plants of Ceylon, which is to be published. One other paper appeared in 1930 (*J. Chem. Soc.* 1930, 2035).

Chandrasena's powerful influence as a teacher has been spoken of elsewhere (*J. Chem. Soc.* September 1934). The writer is glad to take this opportunity of referring to his untiring kindness to his colleagues in other Scientific Institutions in Ceylon; he was always interested and helpful in their particular problems, and his unexpected death on June 10th, 1934, has left a serious gap in local scientific circles.

R. C.

JOHN JAMES RICKARD MACLEOD[1].

(1876–1935.)

J. J. R. Macleod was born in the little village of Cluny, near Dunkeld; but his father, the late Rev. Robert Macleod, was shortly afterwards called to a charge in Aberdeen, and it was at the Aberdeen Grammar School and Aberdeen

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[1] I wish to thank my colleague, Dr D. L. Thomson, a native of Aberdeen and an acquaintance of long standing of Prof. Macleod, for his help in furnishing the details of this biographical sketch.—J. B. C.

University that the son received his education. He graduated with honours in medicine in 1898, winning the Matthews Duncan and Fife Jamieson medals; he was also awarded the Anderson Travelling Scholarship, which enabled him to spend the following year in study at the Physiological Institute of the University of Leipzig. He returned to become Demonstrator in Physiology, and eventually Lecturer in Biochemistry, at the London Hospital Medical School; during this period he obtained the Diploma of Public Health at Cambridge, and was elected Mackinnon Research Scholar of the Royal Society. In 1903, however, he became Professor of Physiology at the Western Reserve University, Cleveland, Ohio, and retained this appointment until he went to the chair of Physiology at the University of Toronto in 1918. He had by this time attained an outstanding position in the field of carbohydrate metabolism, and it was both appropriate and fortunate that the discovery of insulin should have been made in his laboratory. Well-deserved honours were showered upon him; he received the 1924 Nobel prize for Medicine, jointly with his collaborator, Doctor (now Sir Frederick) Banting; he was elected a Fellow of the Royal Society in 1923, and awarded the Cameron prize of Edinburgh University; he received honorary degrees from Toronto, Western Reserve, Aberdeen, and Jefferson Medical College in Philadelphia. He retained the strongest affection for his native land, which he often revisited; and in 1928 was glad to accept appointment to the chair of Physiology at the University of Aberdeen, in succession to Prof. J. A. MacWilliam. Here he continued his researches, and stimulated others to investigation on similar lines, both within the University and at the Rowett Research Institute; he also served as an enthusiastic member of the Medical Research Council. He had built a most attractive home at Bieldside, from whose windows he could look up the beautiful valley of the Dee to the outliers of the Grampians, and in whose sunny garden he greatly delighted. Unhappily he fell a victim to an obstinate and crippling arthritis. He bore his disabling and most painful affliction with the greatest courage and fortitude and continued to direct the work of his Department long after he was forced to give up active participation. His health became gradually worse; pleurisy and at last pericarditis set in, and he died at his home on March 16th, 1935. He was married to a compatriot, Mary McWalters, by whom he is survived.

Macleod's first contributions to physiology were studies on the intracranial circulation (a subject to which he later returned) and on caisson disease, which were partly carried out in conjunction with Sir Leonard Hill. In his first years at Western Reserve he published a series of papers on the carbamates and one on purine metabolism. In 1907 there appeared the first of a long series of "Studies in Experimental Glycosuria", in the *American Journal of Physiology.* These in fact resolve themselves into a series of studies on the breakdown of liver glycogen, whether produced by piqure, stimulation of the splanchnics, reflexly, by asphyxia, or by injection of adrenaline. He probably owed to his year in Germany a vivid conception of the problem as one fundamentally involving the access of the diastatic enzyme to the stored glycogen and was at pains to show that fluctuations in the amount of enzyme present need not be taken into consideration. He became convinced that stimulation of the sympathetic supply, while it doubtless liberated adrenaline whose efficacy in accelerating glycogenolysis was indubitable, also affected the hepatic cells directly. In the later years of this epoch the increase in lactic acid production by the muscles in presence of experimental alkalosis was convincingly demonstrated.

In his first years at Toronto he studied the peculiarities of respiration in decerebrate animals and of the effects of anoxaemia and of excess oxygen thereon.

However, when Banting and Best, to whom he had accorded the facilities of his laboratory, succeeded in their attempts to demonstrate reduction of the hyperglycaemia and glycosuria of depancreatised dogs after injection of crude extracts of degenerated or of foetal pancreas, he returned to the problems of carbohydrate metabolism with renewed enthusiasm. The story of this collaborative research, which at length made insulin available in a form suitable for clinical and experimental use, has often been told. Macleod immediately took up the task of unravelling the mechanism whereby insulin reduced the blood sugar level and was at once able to dispose of obvious but erroneous theories, such as increased glycolysis within the circulation. That the action of insulin was at least not wholly hepatic was shown in his laboratory by Hepburn and Latchford, who demonstrated an effect of insulin on the sugar consumption of the isolated rabbit heart. With the technique at his disposal, it was not possible for him to account for the disappearing sugar in terms of increased oxidation and glycogen formation, as was subsequently done by Best, Dale, Hoet and Marks.

Macleod, influenced by his earlier studies with Pearce on the disappearance of sugar from the circulation of the eviscerated animal even in the absence of the pancreatic hormone, rejected the view that the oxidation of carbohydrate is seriously impeded in pancreatic diabetes, in favour of the view that in this state there is excessive production of glucose from protein and (since the observed protein metabolism is inadequate to account for the flooding of the body with glucose, if the oxidative destruction of carbohydrate is assumed to proceed unchecked) from fatty acids. This attitude involved regarding the respiratory quotient as made up of two components, representing hepatic glyconeogenesis and peripheral carbohydrate oxidation, and rejecting as fictitious the alleged constancy of the urinary dextrose-nitrogen ratio in the fasting diabetic animal. It at once brought him into conflict with the majority of American physiologists, headed by the late Graham Lusk. He obtained further evidence for his stand in careful studies, especially with Chaikoff, on dogs which had recovered from the trauma of pancreatectomy under insulin treatment and were then allowed to pass into diabetes; such animals, he maintained, displayed most variable dextrose-nitrogen ratios, and showed elevated respiratory quotients during exercise (shivering). In his discussion of insulin in *Physiological Reviews*, and in the monograph *Carbohydrate Metabolism and Insulin*, Macleod's extreme position is less apparent than in the later lectures published under the title *The Fuel of Life*, in which he felt that he was not speaking *ex cathedra*. Macleod's position in this difficult question has been loyally upheld by many of his pupils; but it is not improbable that the truth may lie in a compromise between the two extreme views, admitting some restriction of carbohydrate metabolism on the one hand, and increased glyconeogenesis, from protein at least, on the other.

One very important contribution was the discovery, by Karczag, Macleod and Orr (1925), that with proper precautions the liver and muscle glycogen stores of inbred rats could be brought by fasting to a level predictable with some accuracy; this made possible the use of rats for studies on glycogen distribution after insulin injection, or in other experimental conditions, and led to valuable studies by Macleod and his collaborators, and elsewhere, especially by Cori and Cori at Buffalo.

After his translation to Aberdeen, Macleod collaborated with workers at the Rowett Institute on problems of the specific rates of absorption (from the intestine) of various sugars and drew attention to the part played by the stomach in retaining highly concentrated solutions until considerable dilution had occurred. At the University he, with Peterson and others, resumed the study of the metabolism of the decerebrate eviscerated animal, with special reference to the respiratory quotient. His last important publication, with Donhoffer, marks a return to one of his early problems, the nervous control of the glycogenic function of the liver; they sought to show that the hyperglycaemia which follows trauma to the pons, in the rabbit (a modified piqure), was not wholly explicable in terms of adrenaline discharge, nor indeed in terms of hydrolysis of preformed glycogen at all, but in some cases at least indicated a stimulation of hepatic glyconeogenesis by the parasympathetic innervation.

In 1903 Macleod wrote a *Practical Physiology,* which is now little known. His later text-book, *Physiology and Biochemistry in Modern Medicine*, achieved greater success; it was distinguished from the start by a character which it still possesses, though the recent seventh edition is in the form of a symposium: that physiological and biochemical subjects are treated with equal care and mastery, and that the application of these studies to medicine is indicated without being over-emphasised.

Macleod was distinguished by cheerfulness, kindliness and patience; he was always willing to discuss the problems of his juniors sympathetically, and without a trace of condescension. It was typical of him that he would not allow an enthusiastic colleague or assistant to embark on an investigation without pointing out all the technical or theoretical difficulties which would probably be encountered, and yet would supply encouragement and all the practical assistance at his disposal.

J. B. C.

CLII. THE PERIPHERAL ACTION OF INSULIN IN NORMAL ANIMALS.

By NELLES BOYD LAUGHTON
AND ARCHIBALD BRUCE MACALLUM.

From the Departments of Physiology and Biochemistry, University of Western Ontario, Faculty of Medicine.

(*Received March 14th, 1935.*)

SINCE the discovery of insulin little advance has been made in knowledge of its mode of action and of the changes which it brings about in the tissues.

Macleod [1934], summarising the work of the past 15 years, concludes that the conversion of blood sugar into muscle glycogen is not a conspicuous feature of insulin action in normal animals. In so far as oxidation of glucose in the tissues is responsible for the fall in the blood sugar, attention is called to the demonstration by Cori [1931] of a relationship between sugar disappearing from the blood and sugar metabolised after administration of insulin to normal animals. Macleod cites experiments by himself and collaborators which show that the respiratory quotient does not rise during the period when the blood sugar is falling, but only when the convulsive level of hypoglycaemia is reached, indicating that increased oxidation is not responsible for the lowering of blood sugar in the early stages of insulin action. Since it requires only the removal of 75 mg./100 ml. to reduce the blood sugar to the convulsive level in a rabbit, Macleod believes that his hypothesis, that insulin acts by converting the glucose into some intermediate form in the tissues still holds good, although the evidence is against this form being hexosephosphate.

A new approach to this problem is now possible as a result of the recent coordination of views on tissue respiration [*cf.* Keilin, 1933] and of conceptions of intermediary carbohydrate metabolism [*cf.* Cori and Cori, 1933].

This method of approach is illustrated in Fig. 1, which indicates the possible correlation of the respiratory system of the cell and the various intermediate stages in the breakdown of glucose to lactic acid. Certain agents are well known to inhibit various stages of cellular respiration in tissue slices and to interfere with intermediate stages in the conversion of glucose into lactic acid by the action of minced muscle preparations, pressed muscle juices and extracts. In Fig. 1 these inhibiting agents are indicated by frames. The arrows extend from the frames to the points where their specific retarding actions are exercised. Ethylurethane and cyanide inhibit dehydrogenase and oxidase respectively [Keilin, 1933], sodium fluoride has been used to prevent formation of pyruvic acid, and iodoacetic acid inhibits the interaction of pyruvic and glycerophosphoric acids [see Pollard and Pryde, 1933]. We have based our conclusions on the assumption that the same processes take place *in vivo* and that massive inhibition at any of the stages indicated in Fig. 1 will interfere with carbohydrate metabolism in the tissues generally, resulting in hyperglycaemia during the period when the inhibitor is active. Such behaviour is known to result from the action of cyanides [Ohmi, 1933, 1, 2] and of iodoacetic acid [Irving, 1934]. The hyperglycaemic action of anaesthetics and some narcotics is well known; as indicated later we have shown that fluorides have a similar effect. It has indeed been shown that insulin can lower the high blood sugar values produced by iodoacetic acid, cyanides and anaesthetics, but massive doses of insulin have been used and the observations have been made by different individuals so that it is impossible to assess any relative values to the insulin-antagonising actions of the above-mentioned compounds. We have therefore attempted to ascertain the point of maximum activity of insulin by using a standard amount of the latter and studying its relative effects on animals which have been given maximum tolerated doses of one of the inhibitors. A preliminary report has already been published [Laughton and Macallum, 1934].

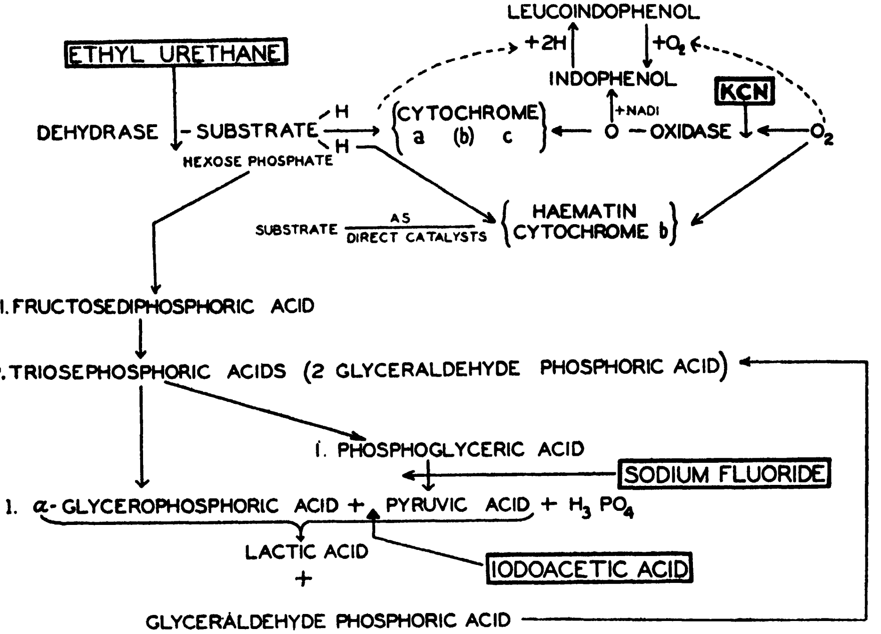

Fig. 1.

During the progress of this study 73 different animals were used. The maximum tolerated dose of each of the compounds was ascertained and records of the blood sugar under its action obtained over a period of time. In other groups of animals the maximum tolerated doses of the respective agents were administered to animals which had received 1 rabbit unit per 2 kg. body weight of insulin 30 or 60 minutes previously, and also in some cases insulin and the inhibitor were administered simultaneously; in a few cases insulin was given 60 minutes after the retarding substance. In all cases the blood sugar values were observed at specified intervals for a period of 3 hours.

Effects of inhibitors on action of insulin.

Cyanide. Curve 1, Fig. 2, represents the effect of 1 rabbit unit of insulin per 2 kg. on a normal rabbit. Curve 3 shows the blood sugar curve of a normal rabbit to which 1·2 mg. of KCN per kg. were administered intravenously, while curve 2 indicates the effect on the blood sugar of a rabbit of the standard dose of insulin at the beginning of the experiment followed 30 minutes later by intravenous injection of 1·2 mg. of KCN per kg. As indicated by the immediate rise in blood sugar, cyanide exercised a temporary inhibiting effect on the insulin which was comparable in duration to its hyperglycaemic effect in the normal rabbit.

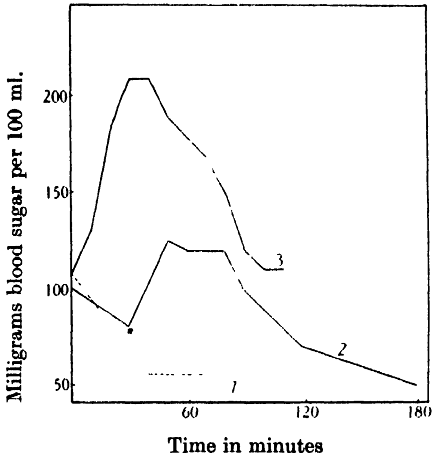

Fig. 2.

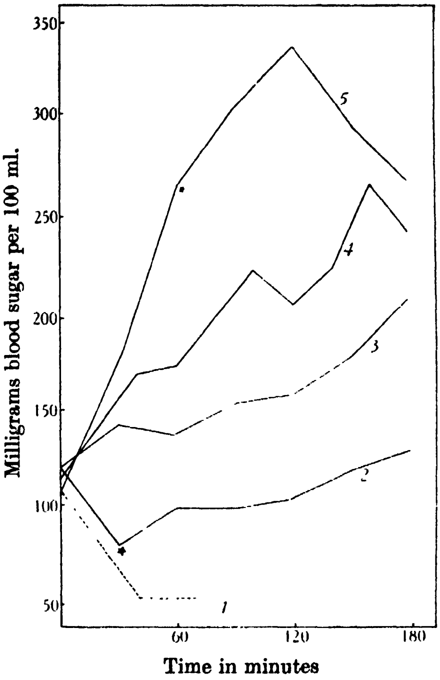

Fig. 3.

Fig. 2. 1. Insulinised rabbit; 2. Insulinised rabbit. *Cyanide injected; 3. Cyanide hyperglycaemia.

Fig. 3. 1. Insulinised rabbit; 2. Insulinised rabbit. *Urethane administered. 3. Insulin and urethane administered simultaneously; 4. Urethane hyperglycaemia; 5. Urethane hyperglycaemia. *Insulin administered.

Ethylurethane. Curve 1, Fig. 3, shows the normal action of the standard dose of insulin. Curve 2 shows the blood sugar values following the administration of the standard dose of insulin at the beginning of the experiment, followed 30 minutes later by intraperitoneal injection of 2·5 g. urethane per kg. (15 % soln.). Curve 3 gives the effect of simultaneous injection of the standard doses of insulin and urethane at the beginning of the experiment. Curve 4 shows the characteristic hyperglycaemia following the administration of the standard dose of urethane alone. Curve 5 indicates the effect of the standard amount of urethane given at the beginning of the experiment followed 60 minutes later by the standard dose of insulin.

Fluoride. Here again Curve 1 is the effect on a normal rabbit of the standard dose of insulin. Curve 3 shows the influence of 75 mg. of NaF (5 % soln.) given intraperitoneally and curve 2 that of the standard dose of insulin followed 60 minutes later by 75 mg. of NaF per kg.

Iodoacetic acid. Curve 1 shows the effect of the standard dose of insulin. Curve 2 gives the blood sugar values in a rabbit which was given the same dose of insulin followed 60 minutes later by intraperitoneal injection of 35 mg. of iodoacetic acid (1 % soln.) per kg., and curve 3 those of an animal which received 35 mg. iodoacetic acid per kg. and 60 minutes later the standard dose of insulin. Curve 4 represents the changes in blood sugar resulting from the administration of 75 mg. of iodoacetic acid per kg. at the beginning of the experiment.

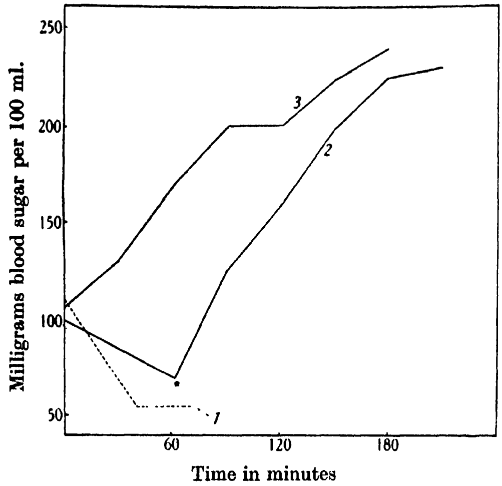

Fig. 4.

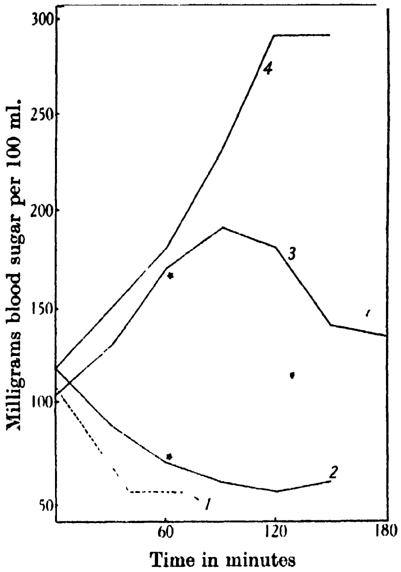

Fig. 5.

Fig. 4. 1. Insulinised rabbit; 2. Insulinised rabbit. *Fluoride administered; 3. Fluoride hyperglycaemia.

Fig. 5. 1. Insulinised rabbit; 2. Insulinised rabbit. *Iodoacetic acid administered; 3. Iodacetic acid hyperglycaemia. *Insulin administered; 4. Iodoacetic acid hyperglycaemia.

Discussion.

It will be noted that the inhibitors by themselves all produce marked hyperglycaemia. It is possible and probable that this effect is caused by inhibition of glucose breakdown at the points indicated in Fig. 1. In the case of cyanide it is obvious that insulin does not counteract the hyperglycaemic action (curve 2, Fig. 2), but that the hypoglycaemic action of insulin is delayed and reappears after the effect of cyanide has passed off. In the presence of insulin the duration of the inhibiting action of cyanide approximates to the duration of its hyperglycaemic action in the normal animal.

In the case of urethane the animals were deeply narcotised and the effect persisted throughout the duration of the experiment (curve 4, Fig. 3). It is also shown by curves 2, 3 and 5 that the urethane effect inhibited the usual action of insulin (curve 1).

In the animals treated with sodium fluoride steadily increasing hyperglycaemia was noted upon which insulin was without influence.

The most striking effects were found after administration of iodoacetic acid. As seen in curve 2, Fig. 5, where iodoacetic acid was administered 60 minutes after the standard dose of insulin, the hypoglycaemic action of insulin still persisted although it was somewhat delayed. The effect of insulin was again demonstrated (curve 3, Fig. 5) when it was injected 60 minutes after the standard amount of iodoacetic acid; here the lowering of blood sugar was not immediate, but in the course of an hour there was a distinct drop in the hyperglycaemia in direct contrast to the effect shown in curve 4 which followed the administration of iodoacetic acid alone.

A survey of the previously noted results indicates that insulin exercises its maximum activity at the point where pyruvic acid reacts with glycerophosphoric acid to form lactic acid and glyceraldehydephosphoric acid [see Pollard and Pryde, 1933]. It is also evident that interference with cell respiration (urethane and cyanides) lowers the efficiency of insulin and that sodium fluoride, which prevents the formation of pyruvic acid from glyceraldehydephosphoric acid is antagonistic to insulin. These results must be considered on a strictly relative basis since it is well known that the hyperglycaemia of narcosis and anaesthesia can be reduced by massive doses of insulin.

The principal criticism to be advanced against the preceding conclusions is that the hyperglycaemia is due to rapid mobilisation of liver glycogen rather than to retardation of tissue metabolism.

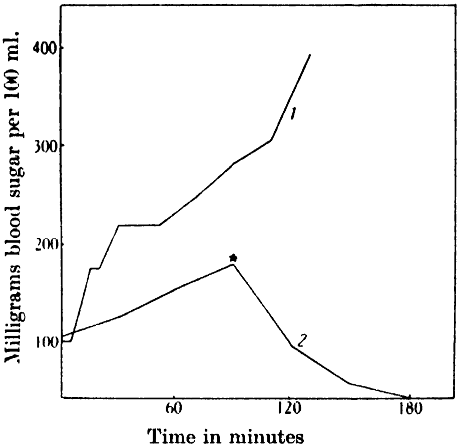

Fig. 6. 1. Adrenaline hyperglycaemia; 2. Adrenaline hyperglycaemia. *Insulin administered.

This criticism can be refuted by the experiment shown in Fig. 6 which illustrates the effect of the standard dose of insulin on adrenaline hyperglycaemia. In this series of experiments the peripheral metabolism was intact and here we see in curve 1 the characteristic response to adrenaline alone (1 ml. of 1:10,000 per kg.). In the experiment illustrated by curve 2, 90 minutes after the adrenaline was given, the rabbit received the standard dose of insulin used throughout the course of this work as a result of which there was a characteristic hypoglycaemic reaction terminating in a convulsion 90 minutes after the injection of insulin. Similar results were obtained in other animals in this series. We may then conclude that the amounts of insulin which have been used in the course of this work will effectively control hyperglycaemia originating from mobilisation of liver glycogen and consequently it is both possible and probable that the hyperglycaemias following the actions of the inhibitors are due to the specific actions of the latter in the respiratory cycle of the tissues and in the intermediate stages of glucose breakdown; this view has already been expressed by Irving [1934] in regard to iodoacetic acid. These experiments tend to support Macleod's view that insulin converts the sugar into an intermediate form in the tissues.

SUMMARY.

1. The action of maximum tolerated doses of cyanide, urethane, iodoacetic acid and sodium fluoride produces a hyperglycaemia which, except in the case of iodoacetic acid, neutralises, at least temporarily, the action of 1 rabbit unit of insulin per 2 kg. body weight.

2. Iodoacetic acid in maximum tolerated doses delays but does not neutralise the effect of 1 rabbit unit of insulin per 2 kg. body weight.

3. Insulin exercises its maximum effect upon the reaction between pyruvic and glycerophosphoric acids.

4. Mobilisation of liver glycogen is not the sole factor in the hyperglycaemias resulting from the substances used in the preceding experiments.

REFERENCES.

Cori (1931). *Physiol. Rev.* **11**, 143.
—— and Cori (1933). *Ann. Rep. Biochem.* **3**, 151.
Irving (1934). *J. Physiol.* **80**, 360.
Keilin (1933). *Ergebn. Enzymforsch.* **2**, 239.
Laughton and Macallum (1934). *Nature*, **134**, 325.
Macleod (1934). *Bull. Johns Hopkins Hosp.* **54**, 79.
Ohmi (1933, 1). *Tohoku J. Exp. Med.* **21**, 107.
—— (1933, 2). *Tohoku J. Exp. Med.* **21**, 115.
Pollard and Pryde (1933). *Chem. Soc. Ann. Rep.* **30**, 330.

CLIII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

VII. THE DISAPPEARANCE OF LYSIN DURING STROMATOLYSIS.

BY ERIC PONDER.

From the Biological Laboratory, Cold Spring Harbor.

(*Received March 15th, 1935.*)

THE observations to be described in this paper are related to others which have been recently published on the disappearance of lysin during the process of haemolysis. Since lysin disappears during the latter process, presumably as a result of its combining with some component of the cell envelope [Ponder, 1934, 1], it is not surprising to find that the disappearance continues after haemolysis is complete, and during that period in which the cell envelopes are being further destroyed (stromatolysis). A study of the later stages of the process, however, reveals certain unexpected relations.

METHODS.

These are an extension of methods already described [Ponder, 1934, 1] and can be summarised as follows.

A suspension of thrice washed red cells is prepared, the cells from 1 ml. of blood being finally suspended in 20 ml. of 1 % NaCl. A suspension of stromata is also prepared from the blood of the same animal, the stromata being obtained by freezing and thawing, with subsequent washing until all haemoglobin is removed. This suspension is diluted so that 0·4 ml. of it is exactly equivalent, so far as its power to combine with lysin is concerned, to 0·4 ml. of the red cell suspension.

To find that quantity of lysin, *e.g.* saponin, which has disappeared from a system at the end of any time t_1, 0·4 ml. of the equivalent stroma suspension is allowed to react with 1·6 ml. of a given dilution of the lysin for this time at a constant temperature (30° in the following experiments, except where otherwise stated). At the end of the time t_1, 0·4 ml. of the red cell suspension is added to the system and the time t_2 required for its complete haemolysis determined. From a suitable time-dilution curve, the quantity of lysin which corresponds to t_2 is found, and this is the amount of lysin left free in the system at time t_1. Subtraction gives x, the quantity which has disappeared. This quantity can be found by this method for any initial concentration of lysin which is not so great as to give exceedingly rapid lysis or so small as to give values of t_2 in excess of about 60 minutes, and also for any value of t_1, from values much less than that which would correspond to complete lysis of 0·4 ml. of red cell suspension by the lysin initially present, up to values very much greater.

A detailed description of the procedure will be found in the paper referred to above, and it is enough to say that the results to be described here were obtained with cell and stroma suspensions made from the blood of both the rabbit and of man. The lysin used was saponin throughout, principally because its disappearance follows a regular course as haemolysis and subsequent stromatolysis proceed. Digitonin, the bile salts and the soaps, all show a rapid "initial absorption" at the cell surfaces, and only when this is complete do these lysins disappear in a regular manner; digitonin, moreover, is difficult to work with because of the high order of its time-dilution curves, and the bile salts and the soaps tend to be unstable.

EXPERIMENTAL RESULTS.

The course of the disappearance.

It can readily be shown that the disappearance of free saponin from a system containing stromata does not cease at a time corresponding to the complete lysis of an equivalent number of red cells but continues at a slower rate for a long time thereafter. This is shown in Table I and in Fig. 1.

Table I.

Initial lysin, γ	"Complete lysis"	γ lysin disappeared after 30 mins.	90 mins.	180 mins.	300 mins.	900 mins.
400	(30)	80	100	130	—	170
200	30	60	70	82	85	90
100	30	46	50	51	53	54
67	31	34	36	36	37	37

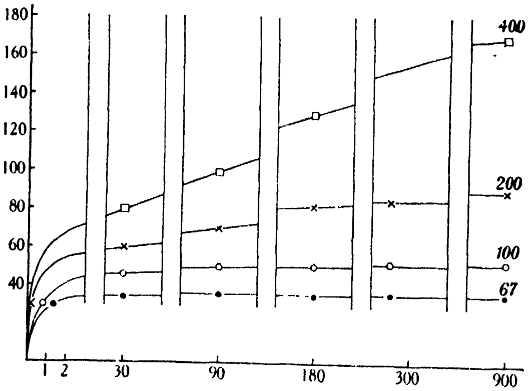

Fig. 1. Ordinate, γ of lysin used up; abscissa, time in mins. The scale on the abscissa has been broken up in order to show the general course of the curves. Squares, initial lysin concentration of 400γ; crosses 200γ, circles 100γ, dots 67γ.

Table I and Fig. 1 show (*a*) that after the lysin and stromata have reacted for a time which would just be necessary for haemolysis of 0·4 ml. of the equivalent red cell suspension ("complete lysis"), a constant amount of lysin has disappeared from the system, in this case 30γ [*cf.* Ponder, 1934, 1], and (*b*) that afterwards there is a continuous disappearance of lysin, the quantity which disappears tending to reach a final value asymptotically. The time it takes for the final value to be approached varies with the initial concentration of the lysin, for when the latter is small the final value is reached quite rapidly, whereas when it is great the concentration of lysin can be shown to be still falling even after many hours. Further, the amount of lysin which ultimately disappears is a function of the initial concentration and an almost linear function moreover, at least within the comparatively small range in which reliable experimental values can be obtained[1]. For very large values of initial lysin concentration the quantity finally removed appears to be less than would be obtained by extrapolating linearly from the values shown in Table I, but with very high lysin concentrations the errors of observation are enormous.

The essential point is that the disappearance of lysin apparently ceases while there is yet a large quantity of free lysin present in the system, and not, as has been supposed hitherto, when the concentration of free lysin has fallen to zero. It is unsafe, however, to attempt to determine the exact form of the curves in Fig. 1, for at least two reasons. (1) There is doubt regarding the reference time-dilution curve which should be used in finding how much lysin is left free in the system. During the early stages of the reaction, when the lysin is being used up rapidly, one must use a reference curve obtained by adding 0·8 ml. of cell suspension to 1·6 ml. of different dilutions of the lysin, for a system in which free lysin is energetically reacting with 0·4 ml. of stroma suspension *plus* 0·4 ml. of cell suspension is more like one in which the lysin is reacting with 0·8 ml. of cell suspension than it is like any other. When the reaction between lysin and stromata has reached equilibrium, on the other hand, the free lysin left reacts only with the added 0·4 ml. of cell suspension, and the proper reference time-dilution curve is one for 0·4 ml. of cell suspension added to 1·6 ml. of lysin *plus* 0·4 ml. of saline. The fact that one has to use one reference curve at the beginning of the reaction and another at the end introduces uncertainty into the units in which the disappearance of lysin is measured. The uncertainty may not be great, for there is not much difference between the two reference curves, but it is great enough to make a detailed analysis of the curves in Fig. 1 almost futile. (2) The results in Table I under the head "complete lysis" are those obtained at times which would be required for complete haemolysis of 0·4 ml. of the red cell suspension by the particular lysin dilution concerned. Now when lysin reacts with a red cell suspension, haemolysing it in time *t*, lysin is used up in reacting both with the cell envelopes (as in the case in which it reacts with a stroma suspension) and also in reacting with liberated haemoglobin; when the lysin reacts with a stroma suspension, on the other hand, there is no pigment and no inhibition, and so more lysin will be used up as a result of a combination with the cell envelopes in the same time *t*. It will be apparent that the quantity which has to unite with the envelopes in order to produce complete lysis cannot be found exactly unless the inhibitory effect of the liberated pigment is allowed for, and this cannot be done quantitatively in the meantime.

Although these difficulties prevent one from analysing the situation fully, there is no doubt as to the departure of the curves from the course which would be expected on the basis of existing theory, and at the same time the experimental results are sufficiently definite to allow the course of the disappearance of lysin to be discussed in a general sort of way. More specifically, it will be observed that the experimental curves are not described by the equations[1]

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[1] For very large initial amounts of lysin, the times for complete haemolysis are too short to be measured exactly, and even such measurements as can be made are complicated by the mixing of the cell suspension and the lysin requiring appreciable time [*v.* Ponder, 1932]. For amounts less than about 67γ, the times for complete lysis may be so great that no measurements can be obtained at all. The experimental range is therefore severely limited and the difficulty in obtaining results correspondingly increased.

$$dx/dt = k\,(c-x)^n \qquad \ldots\ldots(1),$$

or

$$kt = \frac{p}{p-1}\{c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}}\} \qquad \ldots\ldots(2),$$

where

$$p = \frac{1}{n},$$

even if the values assigned to c are those which correspond to the equilibria finally reached (the values in the last column of Table I). Fig. 2 shows an experimental and a calculated curve side by side, and it will be seen that the former falls off much too quickly. The only simple way of accounting for its course is to suppose that the velocity constant of the reaction is continually diminishing, the reaction itself being generally described by expressions (1) and (2). The matter will be discussed more fully below.

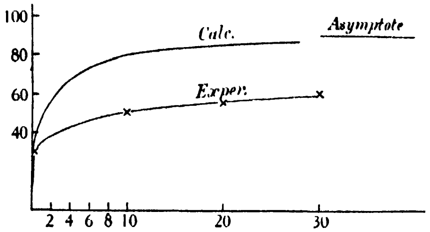

Fig. 2. An experimental curve and a calculated curve obtained from expression (2) with $n = 2{\cdot}0$, both approaching the same asymptote. Ordinate, x in γ; abscissa, time in mins.

The irreversible nature of the combination:

Since the equilibrium conditions are so different from those expected, it is important to test the assumption that the combination between lysin and cell component is irreversible. This is done in the following way.

To 1·6 ml. of a series of saponin dilutions, from about 1 in 5000 to about 1 in 20,000, is added 0·4 ml. of a stroma suspension about five times as concentrated as that used in the above experiments. The tubes are allowed to stand for several hours, at the end of which time the amount of lysin left free is determined. An exactly similar series of tubes is allowed to stand for the same length of time, at the end of which 2 ml. of saline are added to each; 2 ml. of the mixture are removed into a series of clean tubes, and the quantity of lysin left free is found by adding 0·4 ml. of cell suspension, as before. If the amount of lysin left free is exactly half that found in the case of the corresponding lysin dilution in the first series, addition of an equal volume of saline to the systems has done nothing more than dilute the free lysin, and has not caused any of the combined lysin to be liberated again.

The results given by this procedure are shown in Table II. In this experiment the stromata and lysin were allowed to react at 30° for 8 hours. All values are in γ.

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[1] These equations ought to contain terms for the inhibitory effect of liberated haemoglobin on the course of the lytic reaction, but, as the introduction of these terms does not affect the general form of the expressions, the terms have been omitted. See Ponder [1932], and Ponder and Gordon [1934].

Table II.

Initial lysin concentration	Free lysin before dilution	Free lysin after dilution	Ratio
400	160·0	78·5	0·49
200	51·5	25·0	0·49
133	42·0	21·0	0·50
100	37·5	18·0	0·48

The fact that the ratio of free lysin found before and after dilution is so nearly 0·5 shows that the dilution does not cause lysin to be liberated from combination, *i.e.*, that the reaction between the cell component and the lysin is not reversible under these conditions. This is what has always been believed, and it is scarcely necessary to point out that had any other result been obtained the methods used in this and many earlier investigations would have been open to serious objection.

Temperature effects.

The failure of expressions such as equations (1) and (2) to describe the experimental results obtained during the whole course of haemolysis and subsequent stromatolysis is not altogether unexpected, for the expressions themselves have always been unsatisfactory in that they contain an arbitrary constant $n=1/p$. The values assumed by this constant under various conditions have always been puzzling, and it has already been recognised that the introduction of the constant may have no better justification than that it reconciles experimental results with a theory possibly faulty in itself [*v.* Ponder, 1934, 2]. It will suggest itself that the departure of the experimental curves (Fig. 1) from the course expected on the basis of the existing equations may throw light on the meaning of the constant n and its variations, and so I have carried out experiments which attempt to relate the behaviour of the constant to the course of the curves for the disappearance of lysin during haemolysis and stromatolysis. It happens that the easiest way in which n can be varied is by varying the temperature of the haemolytic systems [Ponder and Yeager, 1930].

1. The first step in such an investigation consists in showing that the concentration of the stroma suspension which is equivalent, so far as its power in reacting with lysin is concerned, to a given quantity of red cell suspension, does not itself vary with temperature. The procedure referred to under Methods, above, and already fully described [Ponder, 1934, 1], is repeated at 38, 22 and 7°, three temperatures associated with very different values of n for saponin systems. In each case one begins with a stroma suspension of unknown concentration (because of the variable yield in preparing it), and finds the extent to which it must be diluted so that 0·4 ml. of it is equivalent to 0·4 ml. of red cell suspension. It is necessary to select a dilution of lysin for which results can be obtained at all three temperatures, and so the lysin concentration with respect to which the determinations can be made is exceedingly limited; if the concentration is too great, lysis is so rapid at 38° that the time for complete lysis cannot be observed, and if the concentration is too small, complete lysis never occurs at 7°. This, however, is of little consequence, for the extent to which the stroma suspension has to be diluted seems to be virtually independent of the lysin concentration used to determine it. Table III shows typical results for an experiment in which the dilution of saponin was 1 in 30,000, and the cells and stromata were derived from the rabbit.

Table III.

Required dilution of stroma suspension	Temperature (° C.)
1 in 3·0	38
1 in 3·0	22
1 in 2·8	7

Allowing for experimental error, which in determinations such as these is considerable, these results show that the "equivalence" of the stroma suspension is independent of temperature.

2. The next step is to see whether the amounts of lysin which finally disappear from systems containing stromata and different initial concentrations of lysin are themselves affected by variations in temperature. This is done by repeating experiments such as shown in Table I at different temperatures, 0·4 ml. of equivalent stroma suspension being added to various dilutions of lysin, and the quantity of the latter left free after about 420 minutes being found as already described. For all practical purposes, equilibrium is reached within this time, even at 6°. Table IV shows results obtained for saponin and a rabbit stroma suspension at 38, 20 and 6°.

Table IV.

Initial lysin, γ	γ lysin disappeared after 420 mins. at		
	38°	20°	6°
670	—	420	370
500	—	300	280
400	220	230	210
333	170	190	180
250	130	140	130
200	120	120	120
100	70	70	—
67	51	49	—

Reading across the rows it will be seen that for each initial quantity of lysin the amount which finally disappears is substantially the same irrespective of the temperature, *i.e.*, the equilibrium conditions in these systems are not appreciably affected by temperature. This is interesting in view of the many theories which associate haemolysis *etc.* with an adsorption of lysin by the material of the cell envelope [Clark, 1933; Reiner, 1934], for in adsorption reactions larger amounts are usually adsorbed at lower temperatures. It may also be observed that the equilibrium conditions for the process of haemolysis, which is dependent on the value of x in expressions (1) and (2), are also scarcely affected by temperature. A study of the equilibrium conditions accordingly throws no light on the arbitrary constant n.

3. We can, however, extend the investigation by plotting the course of the curve for lysin utilisation at different temperatures, *e.g.*, at 38° and at 6°. Consider one concentration of lysis, such as 200γ, reacting with stromata at the two temperatures, and imagine the amount of lysin x which disappears plotted on the ordinate against time t on the abscissa (as in Fig. 3). When $t=0$, $x=0$, and when $t=\infty$ both curves pass to the same final value of x (2, above). They may nevertheless pursue entirely different courses between these two points, and on each there is a point of particular interest, *viz.*, the point which corresponds to the time required for the complete lysis of a quantity of red cell suspension equivalent to the amount of stroma suspension with which the lysin is

reacting. The peculiarity of this point lies in the fact that it has already been shown that, at any one temperature, the quantity of lysin used up at the time needed to produce complete haemolysis is constant, irrespective of the initial lysin concentration [Ponder, 1934, 1]. It is therefore important to establish whether or not the amount of lysin used up at the time required for complete lysis of a red cell suspension is constant irrespective of temperature, and at first sight it would appear that this could be done by plotting the curves for the utilisation of lysin in their entirety at the two different temperatures and seeing if both curves pass through points characterised by the same value of x on the ordinate and by values of t corresponding to the times for complete lysis of 0·4 ml. of red cell suspension at the two different temperatures on the abscissa. This, however, is impracticable, for if we select a concentration of lysin which produces complete haemolysis of 0·4 ml. of red cell suspension in a reasonable time at 6°, the time for complete lysis at 38° is so short that small variations from it cannot be measured with certainty. Conversely, if we select a concentration which gives complete lysis at 38° in a time sufficiently long to allow of small variations to be satisfactorily measured, lysis at 6° is not complete even in six or eight hours. We have accordingly to change the lysin concentration when we change the temperature, and to rely on the fact that the amount of lysin utilised at the moment of complete haemolysis is constant at any one temperature, irrespective of the initial lysin concentration. The procedure is best illustrated by a typical experiment.

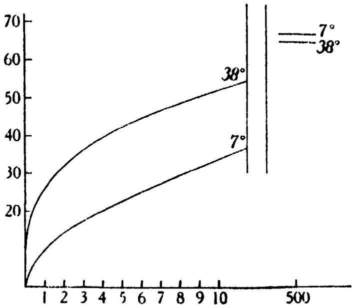

Fig. 3. Curves for the using up of lysin from an initial lysin concentration of 200γ at 38 and 7° respectively. Ordinate, x in γ; abscissa, time in mins. The equilibrium values, reached at about 500 mins. are substantially the same, and are shown by horizontal lines.

Taking 1·6 ml. of 1 in 100,000 saponin at 38° and adding 0·4 ml. of red cell suspension, one gets complete lysis in 11 seconds. Allowing the same amount of lysin to react with 0·4 ml. of equivalent stroma suspension for 11 seconds and then adding 0·4 ml. of cell suspension, one gets complete lysis in 42 seconds. The time required for complete lysis when 1·6 ml. of 1 in 120,000 saponin reacts with 0·8 ml. of cell suspension is 32 seconds, and that required when 1 in 160,000 saponin reacts under the same conditions is 90 seconds. Interpolating, the time of 42 seconds corresponds to a dilution of 1 in 134,000. Converting to the number of γ of lysin in the system of volume 2·4 ml., the initial amount of lysin is 40γ, and the amount left free at the end of 11 seconds is 30γ. The difference, x, is 10γ.

Repeating the same experiment at 6° with a lysin dilution of 1 in 1000, complete lysis of the 0·4 ml. of cell suspension occurs in 1·0 minute. Allowing the lysin to react with 0·4 ml. of equivalent stroma suspension for 1·0 minute, and then adding 0·4 ml. of cell suspension, we get complete lysis in 1·35 minutes. This corresponds to a lysin dilution of 1 in 10,300 reacting with 0·8 ml. of red cell suspension. Converting to γ, the initial amount of lysin is 400γ, and the amount left free after 1·0 minute is 389γ. The difference is 11γ, which is substantially the same as the difference obtained at 38°.

Experiments of this kind leave no doubt as to the constancy of the quantity of lysin which disappears in the time required for complete haemolysis, and that the amount appears to be independent of both temperature and initial lysin concentration. The determinations, of course, have a considerable experimental error attached, but the consistency with which the same result is obtained is, all things considered, remarkable.

4. Table V shows the result of an experiment in which the curves for the disappearance of lysin reacting with stromata are obtained in their entirety at 38 and at 7°. The lysin concentration used was 200 γ, and the times are in minutes.

Table V.

38°		7°	
t	x	t	x
0·0	0	0·0	0
0·017	(15)	2·2	15
2·2	33	5·0	23
10·0	52	10·0	34
500·0	65	500·0	67

Assuming equilibrium to have been reached in 500 minutes, both curves pass to the same equilibrium value (*cf.* 2, above). The point (0·017, 15) has been inserted in order to make the amount of lysin which disappears in 1 second at 38° and the time required for complete lysis of 0·4 ml. of red cell suspension at that temperature, correspond to the 15 γ of lysin used up in 2·2 minutes at 7°, this being the time required for complete lysis at 7°. The results are plotted in Fig. 3, and it will be seen that the two curves approach the same equilibrium value in quite different ways. They are like the curves of Fig. 1 in that neither can be described by expressions (1) and (2) with a constant value of k, but their course throws some light on the value of n in these equations, for the curve at 38°, with its sharp curvature, bears a general resemblance to a curve described by expression (2) with a value of n of about 3·0, while the flatter curve at 7° is not unlike that for a first order reaction, *i.e.*, a curve described by expression (2) with $n = 1{\cdot}0$. The variations of the value of n which have already been described as a result of changes in temperature are accordingly reflected in the form of the curve for the utilisation of lysin at various temperatures; at high temperatures, the value of n in the existing equations is 2·5 or greater, and the curve for the utilisation has a high degree of curvature, while at low temperatures the value of n approaches 1·0, and the curve for the utilisation of lysin is relatively flat.

DISCUSSION

The experimental curves for the process of haemolysis followed by stromatolysis differ from those expected on the basis of existing theory in two ways. (1) The equilibrium conditions are different, the using up of lysin ceasing while considerable quantities of lysin are still free in the system, and not when the free lysin concentration has fallen to zero. (2) The course of the curves for the utilisation of lysin cannot be described by the existing equations unless the value of the velocity constant k is imagined to vary. In considering the effect of these two observations on existing theory, it is convenient to treat them separately.

1. As shown in Tables I and IV, the amount of lysin which finally disappears as a result of reacting with the stromata is very nearly a linear function of the initial lysin present. Let the slope of the line be a. Rewriting (1) to express the idea that the velocity of the reaction is dependent on the quantity of lysin in excess of the equilibrium value, we have

$$dx/dt = k\,(ac - x)^n \qquad \text{......(3)},$$

which gives on integration

$$kt/a^{\frac{1-p}{p}} = \frac{p}{p-1}\{c^{\frac{p-1}{p}} - (c - x/a)^{\frac{p-1}{p}}\} \qquad \text{......(4)}.$$

Comparing this expression with expression (2), it will be clear that the two equations can be made to give the same values of t by multiplying every value of c in expression (4) by $1/a$, and changing the value of the velocity constant. Since the only way of measuring x is in terms of c and in units which are really arbitrary, and since there is no independent way of finding the value of the velocity constant of the reaction, the two equations are identical in so far as numerical results are concerned. The fact that the equilibrium conditions are other than those postulated in the existing theory accordingly makes no difference; this, of course, is wholly due to the relation between the values of x at equilibrium being linear with the initial values of c.

2. The fact that the course of the curves for the utilisation of lysin cannot be described by expression (2) with a constant value of the velocity constant, and therefore cannot be described by expression (4) either, leads to the unescapable conclusion that the existing theory is wrong in some fundamental respect. There are, of course, many ways in which it may be at fault, but the most obvious of these is that it supposes the velocity constant of the reaction to have the same value at all stages of the reaction, whereas in fact it may be steadily decreasing. On the basis that it is undergoing a continuous decrease it would obviously be possible to fit the experimental curves of Figs. 1 and 3 with a first order equation, or with an equation of any order less than that required to fit the time-dilution curve for haemolysis under the same conditions and by the existing equations, provided the velocity constant were allowed to decrease appropriately. If the possibility of such a decrease in k is once admitted, however, there is no justification for supposing that the decrease occurs only during stromatolysis and after haemolysis is complete; one might as well suppose it to begin to decrease from the very beginning of the process, and describe the entire course of the disappearance of lysin during both haemolysis and subsequent stromatolysis by a first order equation, together with an appropriate decrease in k as the reaction proceeds. Under such circumstances the meaning of the constant n in equations (2) and (4) would be clear, and this constant would merely express, in an arbitrary sort of way, the extent of the fall in the value of k during the course of haemolysis. For example, if the reaction were really of the first order, and n were 1·0, the value of k would not decrease at all, but if n were 2·0 and the reaction of the first order, k would have to decrease as the reaction proceeded, while if n were 3·0 the decrease would have to be greater. Such a hypothesis would, of course, leave us with no difficulty in accounting for the non-integral values which the constant n usually assumes.

Reaching a conclusion like this, of course, is tantamount to admitting that the equations hitherto used for describing the course of haemolysis are merely formal ones. In fact, it raises the disturbing doubt as to whether correct equations for the kinetics of any biological system can ever be formulated except in cases in which the behaviour of the velocity constant is first defined exactly, and such cases appear to be rare at the present time. The possibility of a mathematical description of the kinetics of haemolysis and stromatolysis, other than a purely formal one, at all events becomes rather remote[1].

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[1] The reader will observe that, although the equations hitherto used to describe time-dilution and percentage haemolysis curves are probably only formal ones, the system of analysis used by myself and my collaborators in previous papers requires no essential modification. This is due to the analysis being based on the comparison of time-dilution and percentage haemolysis curves, obtained under one set of conditions, with similar "standard" curves obtained under simpler conditions. So far as the method of analysis is concerned, the experimental curves need never have been described by equations at all.

In conclusion, it ought to be remarked that the type of reaction which now appears to be responsible for haemolysis and stromatolysis is not a unique one. A reaction characterised by virtual irreversibility, a steadily decreasing velocity constant and an equilibrium which is reached when there is still free reactant in the system is met with in a number of processes, such as the dyeing of wool *etc.*, and is known as "pseudo-adsorption" or "anomalous adsorption", terms which mean little except that the reaction is not a stoichiometric one. In all such cases, as in the case of the reaction which results in haemolysis *etc.*, the decrease in the velocity constant is probably associated with the fact that the proper form of expression (1) is

$$dx/dt=k'.F\ (c-x).F\ (S-x) \qquad \ldots\ldots(5),$$

where S is the reacting surface of the substance which is acted on, and that the expression has to be simplified by writing $k=k'.F\ (S-x)$. Obviously, were $F\ (S-x)$ to equal $F\ (c-x)$, we would get expression (1) with $n=2$, but since we know nothing as to how the reacting surface decreases as the reaction proceeds, the whole problem is indeterminate.

SUMMARY.

This paper is concerned with the rate at which a lysin such as saponin is used up during the process of stromatolysis, or breaking down of the red cell envelopes, which follows haemolysis. It is shown that the equations which have hitherto been used to describe the using up of lysin during haemolysis are not applicable to the subsequent process. The contents of the paper do not lend themselves to detailed abstraction, but the principal conclusions are (*a*) that the reaction between cells and lysin is characterised by peculiar equilibrium conditions, in which using up of lysin ceases while there is still free in the system a quantity of lysin which is nearly linear with the amount initially present, (*b*) that the course of the disappearance of lysin is probably described by an equation, the exact form of which cannot be defined, in which there is a velocity constant which undergoes continual decrease as the reaction proceeds, (*c*) that such a decrease in the velocity constant is probably responsible for our having to introduce an arbitrary constant n into the existing equations for the kinetics of haemolysis, and (*d*) that although these existing equations are probably only formal ones, the conclusions based on the existing analysis of time-dilution and percentage haemolysis curves are themselves essentially unchanged.

REFERENCES.

Clark (1933). The mode of action of drugs. (Edinburgh.)
Ponder (1932). *Proc. Roy. Soc. Lond.* B **110**, 1.
—— (1934, 1). *Proc. Roy. Soc. Lond.* B **116**, 282.
—— (1934, 2). The mammalian red cell and the properties of haemolytic systems. Protoplasma Monographien, No. 6 (Gebrüder Borntraeger, Berlin).
—— and Gordon (1934). *Biochem. J.* **28**, 748.
—— and Yeager (1930). *J. Exp. Biol.* **7**, 390.
Reiner (1934). *J. Gen. Physiol.* **17**, 409.

CLIV. THE ANTIHAEMORRHAGIC VITAMIN OF THE CHICK.

By HENRIK DAM.

From the Biochemical Institute, University of Copenhagen.

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Previous papers [Dam, 1929; 1930; Dam and Schönheyder, 1934] deal with a deficiency disease resembling scurvy in chicks which cannot be prevented by ascorbic acid and the cause of which is ascribed to the lack of a particular antihaemorrhagic factor (or factors) in the diet. Schönheyder [1935] has shown that there is an enormous retardation of the clotting of the blood of chicks suffering from this haemorrhagic diathesis.

The nature and distribution of the antihaemorrhagic factor have now been investigated. The investigation has led to the discovery of the fact that the factor is a fat-soluble vitamin occurring in hog-liver, hemp seed, certain cereals and vegetables, and must be different from vitamins A, D and E. It is proposed to term this factor *vitamin K* (Koagulations-Vitamin in German and the Scandinavian languages).

The following groups of foods have been tested: (1) cereals and seeds, (2) vegetables, (3) animal organs, (4) different fats and oils, (5) hen's egg.

Two of the most active substances, hog-liver and hemp seed, were divided into ether-soluble and ether-insoluble fractions, and, since the active principle was found to be fat-soluble, an elaborate fractionation of hog-liver fat was carried out. The question of the identity of the antihaemorrhagic factor with already known fat-soluble vitamins has been attacked by adding large amounts of vitamins A, D and E to the basal diet.

EXPERIMENTAL.

The animals (White Leghorns) were fed the experimental diets from the day after hatching. They were, as a rule, killed at the age of about 1 month. Housing *etc.* as described by Dam and Schönheyder [1934].

The examination of the animals includes, in addition to an inspection of the gizzard and other organs for haemorrhages, a determination of the time of clotting of the blood. The haemoglobin content of the blood has also been determined because it may give some information about the loss of blood through the haemorrhages.

The haemoglobin values were determined by the Sahli haemoglobinometer; the figures indicate the haemoglobin content in percentage of that in normal human blood.

The clotting time was determined by opening the brachial vein by a slight cut and allowing 2–3 ml. blood to drop slowly, during ½–¾ min., into a small porcelain bowl. The clotting time is reckoned from the venepuncture until complete clotting[1].

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[1] Autopsy and clotting time determinations were kindly made by Dr F. Schönheyder to whom the author is indebted. Dr Schönheyder has in the meantime developed a new technique which enables the quantitative determination of the antihaemorrhagic vitamin to be made by a curative method based on a particularly exact measurement of the clotting time [1935]. The results presented in this paper were obtained by means of the former approximate method but give fairly accurate information about the degree of sickness of the animals.

Basal diets.

60		111		124	
Caseinogen vitamin A-free	20	Caseinogen vitamin A-free	20	Ether-extracted dried hog-liver	20
Dried yeast	15	Dried yeast	40	Dried yeast	15
Salts No. 2*	2·7	Salts No. 2	2·7	Salts No. 2	2·7
Sucrose	62·3	$CaCO_3$	0·85	Sucrose	62·3
		Sucrose	36·45		
	100		100		100
Cod-liver oil	4	Cod-liver oil	4	Cod-liver oil	4

* Dam and Schonheyder [1934].

Cereals and seeds were crushed and given instead of sucrose in diet 60 (with a small amount of $CaCO_3$ added roughly to balance the excess P_2O_5 in the cereals). *Organs* were dried in a current of air at a temperature not exceeding 45° and given as a powder instead of caseinogen in diet 60. *Vegetables* were minced and mixed into the diet or given in separate food containers. *Vitamin concentrates:* "vogan" (a vitamin A preparation from Merck, Darmstadt with 120,000 International Units per ml.), "vigantol" (pure vitamin D in sesame oil, from Merck, with 15,000 I.U. per ml.) and carotene (Hoffmann La Roche, Basel) dissolved in olive oil, were given *per os* by means of a tuberculin syringe, the doses being gradually increased from week to week. Vigantol was diluted with olive oil because the day doses otherwise would have been too small for accurate measurement with the syringe.

Hog-liver fat was put at the author's disposal by the firm of Medicinalco, Copenhagen. It was, according to the statement of the manufacturer, prepared by percolation of the desiccated liver with light petroleum several times, and thereafter with ether. The solvents were removed *in vacuo*. The percolated liver was used in diet 124 after having been further extracted with ether in a Soxhlet apparatus for 3 days. Hemp seed oil was prepared by percolating the crushed seeds with ether, and the seeds were further extracted with ether for several days before they were used in the diet as "ether-extracted hemp seed". Wheat germ oil was prepared from the fresh germ which had just been dried in air at 35°. The extraction was carried out in a large Soxhlet apparatus within 8 hours, the bulk of the ether was distilled off in CO_2 and the rest removed *in vacuo*. All oils were stored in the ice-box in completely filled containers and the liver fat *in vacuo*. The oils were used within 2 months of the day of preparation. The ether was in all cases freshly distilled and tested with mercury before use. Non-saponifiable matter of liver fat was prepared by cold saponification of 100 g. of the fat in 250 ml. ether with 40 g. KOH in 250 ml. methyl alcohol for 16–24 hours, addition of water and shaking 3–4 times with more ether; the ether extracts were shaken 3 times with aqueous KOH and then with water to neutral reaction. The extraction and purification of the ether extract were carried out in one day (in the dark room). After standing overnight in the ice-box with Na_2SO_4 and filtering, the solution was concentrated (in CO_2 and then *in vacuo*) to 100 ml. On standing overnight in the ice-box a precipitate of an amorphous substance, which is soluble in ether, could be filtered off. In the experiments in which the sterol and non-sterol fractions were tested, the bulk of the sterols was crystallised from light petroleum (after evaporation of the filtrate from the readily soluble substance). The rest of the sterols were precipitated by digitonin in small excess. The digitonin precipitate was decomposed by the pyridine-ether method of Schönheimer and Dam [1933] in the cold, and the sterol prepared in this way was added to the crystallised main portion. The filtrate from the digitonide was brought to dryness *in vacuo* and extracted with ether which takes up the "easily soluble non-sterol fraction".

The fatty acids were precipitated from the extracted soaps by sulphuric acid after evaporation of the alcohol *in vacuo*; the acids were washed with water by decantation.

In one experiment the non-saponifiable matter was divided into a "carotene" fraction and a "xanthophyll" fraction by partitioning between light petroleum and 90 % (by volume) methyl alcohol, after removing the sterols as far as possible by crystallisation.

Table I.

Chick No.	Age in days when Dead	Killed	Weight g.	Haemoglobin (Sahli)	Time of clotting mins.	Haemorrhages In the lining of the gizzard	Elsewhere
CEREALS AND SEEDS.							
Group 75. 62 % whole wheat:							
564	—	31	127	45	—	+	0
565	—	31	318	65	—	0	0
566	-	31	240	51	—	+	0
567	—	16	87	59	—	?	0
568	—	31	217	59	—	+	0
Group 77. 62 % wheat meal:							
574	—	16	73	48	—	+	0
575	—	31	212	45	—	+	0
576	—	31	224	39	—	+	0
577	—	29	175	37	—	+	+ +
578	—	11	83	48	—	+	+
Group 79. 62 % wheat bran:							
584	—	31	107	49	—	0	0
585	—	31	139	54	·	0	0
586	—	16	55	70	—	0	0
587	-	31	151	52	—	0	0
588	—	31	133	57	—	0	0
Group 86. 62 % wheat germ:							
619	7	—	30	—	—	0	0
620	—	28	174	66	—	0	+
621	—	7	21	—	—	?	0
622	—	33	154	55	—	0	+
623	—	38	183	55	19	+	0
699	—	16	54	—	—	+	0
700	—	30	218	59	2	?	0
Group 93. 62 % oats:							
654	—	33	412	51	2	0	0
655	—	33	442	62	1	?	?
656	—	33	315	62	1	0	0
657	5	—	29	—	—	0	0
658	—	28	234	50	3½	0	+
Group 94. 62 % yellow corn:							
659	—	30	135	42	20	+	+
660	—	33	232	46	—	+	+
661	—	33	179	65	3	+	0
662	—	33	169	35	>12	+	+
663	—	33	195	55	2	+	0
Group 95. 62 % barley:							
664	—	33	285	55	½	0	0
665	—	33	274	58	1	0	0
666	—	33	284	60	½	0	0
667	—	33	225	50	1	0?	0
668	—	33	218	58	½	0	0

Table I (*cont.*).

Chick No.	Age in days when		Weight g.	Haemoglobin (Sahli)	Time of clotting mins.	Haemorrhages	
	Dead	Killed				In the lining of the gizzard	Elsewhere
CEREALS AND SEEDS (*cont.*).							
Group 96. 62 % millet:							
669	—	33	312	60	2½	?	0
670	—	33	290	61	1	0	+
671	—	33	248	61	½	0	0
672	—	33	247	60	½	0	0
673	—	33	304	65	1	+	0
Group 97. 62 % hemp seed:							
674	—	33	258	50	½	0	0
675	—	33	263	60	1	0	0
676	—	33	313	61	½	0	0
677	—	33	310	60	½	0	0
678	—	33	310	50	1	0	0
Group 98. 62 % unpolished rice:							
679	7	—	—	—	—	—	—
680	—	20	154	32	>8	+	+ +
681	—	30	145	45	>12	+	+
682	—	15	75	27	>120	+	+
683	—	13	86	40	>18	+	+
Group 146. 62 % white sunflower seed:							
928	—	31	245	55	>37	+	0
929	—	31	198	58	30	+	0
930	—	31	275	55	1½	+	0
931	—	31	300	55	100	+	+
932	—	31	260	58	5	0	+
Group 147. 62 % black sunflower seed:							
933	13	—	68	—	—	+	+ +
934	—	23	139	55	>130	0	+ +
935	—	16	77	10	>60	+	+ +
936	—	23	146	46	>120	+	+
937	—	23	169	58	>195	0	0
Group 149. 62 % soya bean:							
943	—	33	198	53	½	?	0
944	—	33	147	55	2½	0	0
945	—	33	189	49	2½	+	0
946	—	33	228	50	1	+	0
947	—	33	151	48	2½	+	?
Group 173. 62 % rye:							
1070	—	44	260	42	110	+	+
1071	—	32	220	55	9	+	0
1072	48	—	345	—	—	+	0
1073	—	56	467	54	52	+	0
1074	—	44	265	—	—	+	0
FRACTIONS OF HEMP SEED.							
Group 120. 62 % ether-extracted hemp seed:							
801	—	25	63	50	3½	+	0
802	—	24	52	25	4	+	?
803	19	—	42	—	—	+	0
804	—	9	36	62	2½	0	?
805	—	25	143	—	—	+	0
Group 132. Diet 111 + 25 % hemp seed oil:							
862	—	42	298	50	2	0	0
863	—	42	265	60	2	0	0
864	—	43	310	55	1	?	0
865	—	39	169	45	1	?	0
866	—	33	150	60	1	?	0

Table I (*cont.*).

Chick No.	Age in days when		Weight g.	Haemoglobin (Sahli)	Time of clotting mins.	Haemorrhages	
	Dead	Killed				In the lining of the gizzard	Elsewhere
FRACTIONS OF HEMP SEED (*cont.*).							
Group 111.	Diet 111 without any addition:						
751	—	52	308	35	120	+	+
752	—	52	309	50	6	+	+
753	7	—	—	—	—	0	0
754	—	42	212	32	>26	+	+
755	—	19	114	36	>60	0	+
756	—	45	170	43	>60	+	+
757	—	33	148	25	>60	+	+
VEGETABLES.							
Group 84.	Diet 60 + 25 % fresh kale:						
609	—	9	30	57	—	0	0
610	—	38	218	54	—	0	0
611	—	38	279	51	—	0	0
612	—	32	100	70	—	0	0
613	—	38	200	51	—	0	0
Group 118.	Diet 60 in which 15 % sugar is replaced by dried alfalfa:						
788	—	35	249	52	5½	0	0
789	—	35	204	54	4½	0	0
790	—	35	185	57	½	0	0
791	—	27	150	49	5	0	0
792	—	23	95	53	1½	+	?
Group 104.	Diet 60 without any addition:						
711	—	25	70	<10	60	+	+
712	18	—	54	—	—	+	0
713	52	—	290	34	—	+	+ +
714	—	41	99	—	—	+	+ +
715	—	46	172	10	>12	+	+ +
716	—	33	88	15	10	+	+
717	46	—	158	—	—	+	+
718	—	19	48	<10	>30	+	0
719	—	27	46	<8	>500	+	0
Group 156.	Diet 111 + fresh tomato *ad lib.*:						
978	—	34	190	50	1	0	0
979	—	32	225	50	1½	0	0
980	—	31	133	62	1	?	0
981	—	3	35	–	6½	0	0
982	—	34	125	52	15–3	+	0
Group 164.	Diet 111 + 10 % dried orange peel (outer layer):						
1019	—	23	42	—	2½	0	0
1020	—	34	180	53	2	0	?
1021	7	—	29	—	—	0	0
1022	—	32	182	39	17	0	0
1023	—	34	131	45	1½	0	+
1049	—	25	120	50	3	0	0
1050	—	25	111	50	10–15	0	0
Group 184.	Diet 111 + fresh carrot *ad lib.*:						
1131	—	23	80	48	2	+	?
1132	—	30	155	42	30	0	0
1133	—	30	148	48	3	0	0
1134	—	32	285	43	30	+	0
1135	—	32	208	—	>90	+	+

Table I (*cont.*).

Chick No.	Age in days when		Weight g.	Haemoglobin (Sahli)	Time of clotting mins.	Haemorrhages	
	Dead	Killed				In the lining of the gizzard	Elsewhere
			DRIED ORGANS.				
Group 137.	20 % dried calf-brain:						
887	—	23	92	48	>30	+	+
888	—	30	81	30	>30	+	0
889	—	28	67	40	>60	+	+
890	—	19	66	10	>60	+	0
891	—	31	136	52	>60	0	0
Group 157.	20 % dried ox-muscle:						
984	—	35	130	45	>30	0	0
985	—	23	175	40	6	0	+
986	—	34	210	36	>300	?	+
987	—	24	116	46	12	+	+
988	—	35	220	40	10	0	+
Group 158.	20 % dried adrenals (ox):						
989	—	33	112	55	3½	0	0
990	—	33	112	52	12	0	0
991	6	—	22	—	>10	?	0
992	5	—	28	—	—	0	0
993	—	9	41	50	15	0	0
Group 159.	20 % dried ox-kidney:						
994	—	39	214	48	35	0	0
995	—	39	252	35	4	0	0
996	5	—	38	—	—	0	0
997	—	37	330	48	9½	0	0
998	6	—	40	—	—	0	0
1053	—	39	178	52	12	0	0
1054	—	32	187	44	23	0	0
Group 160.	20 % dried ox-lung:						
999	—	41	172	30	>200	?	+
1000	—	41	150	30	28	+	+
1001	—	29	180	25	>60	0	+ +
1002	40	—	170	—	—	+	+
1003	—	23	164	42	18	0	+ +
Group 161.	20 % dried calf-thymus:						
1004	—	35	132	40	>120	0	+
1005	—	38	191	47	60	0	0
1006	—	38	150	42	30	0	0
1007	—	38	170	51	16	+	+
1008	—	35	68	30	4½	+	0
Group 105.	20 % dried hog-liver:						
721	—	29	133	60	1	0	0
722	22	—	147	—	—	0	0
723	7	—	39	—	—	0	0
724	—	28	177	55	1	0	0
725	—	42	290	43	1	0	0
1164	—	26	140	55	1	0	0
1164*a*	—	31	251	53	16	0	0
1165	—	29	134	48	2	0	0
1166	—	31	154	50	5	0	0
1167	—	31	167	54	4½	0	0
1168	—	28	165	50	1–3	0	?
			FRACTIONS OF HOG-LIVER.				
Group 124.	20 % dried hog-liver extracted with ether:						
821	—	24	150	46	20	+	+
822	—	26	153	50	10	+	0
823	—	18	93	47	>1000	+	+
824	—	27	178	50	>30	0	?
825	24	—	138	52	>60	0	+

Table I (*cont.*).

Chick No.	Age in days when		Weight g.	Haemo-globin (Sahli)	Time of clotting mins.	Haemorrhages	
	Dead	Killed				In the lining of the gizzard	Else-where
FRACTIONS OF HOG-LIVER (*cont.*).							
Group 139. Diet 124 + 3 % hog-liver fat:							
897	—	48	270	48	2	0	0
898	—	47	243	45	3½	0	0
899	—	29	138	50	1½	0	0
900	—	26	120	55	4	0	?
901		45	310	48	2	0	0
Group 125. Diet 124 + 3 % fatty acids from hog-liver fat (corresponding to 4·7 % fat):							
826	—	22	132	52	>30	0	+
827	—	21	134	42	>30	+ ?	+
828	—	23	146	42	5	0	+
829	—	20	110	53	>60	0	+
830	—	21	121	38	>30	0	+
Group 126. Diet 124 + 0·37 % non-saponifiable matter from hog-liver fat (corresponding to 4·4 % fat):							
831	—	29	200	56	14	0	0
832	—	25	152	52	12	0	0
833	—	29	157	53	13	0	0
834	—	29	204	57	2½	0	0
835	—	29	188	59	4	0	0
Group 150. Diet 124 + 0·075 % of a fraction of the non-saponifiable matter sparingly soluble in ether (corresponding to 6 % fat):							
948	23	—	123	—	—	+	+ +
949	24	—	185	—	—	+	+ +
950	—	29	120	35	>60	+	+
951	25	—	120	—	—	+	+
952	—	11	55	52	7	+	+
Group 151. Diet 124 + 0·4 % sterol from hog-liver fat (corresponding to 6 % fat):							
953	—	28	165	22	60	+	+
954	22	—	151	—	—	0	+
955	—	28	136	32	75	0	+ +
956	—	20	132	30	15	0	+ +
957	—	28	105	8	>60	+	+ +
Group 152. Diet 124 + non-saponifiable matter from hog-liver fat freed from sterol and the sparingly soluble fraction (corresponding to 6 % fat):							
958	—	28	220	53	1	0	0
959	—	30	185	52	6	0	0
960	—	30	165	55	2	0	0
961	—	30	195	57	15-6	0?	0
962	—	28	230	55	1	0	0
Group 171. Diet 124 + xanthophyll fraction of the non-saponifiable matter (corresponding to 6 % fat in the first 8 days, thereafter to 12 %):							
1060	24	—	115	—	>30	+	+
1061	—	24	130	45	>45	+	0
1062	24	—	124	—	>60	+	+
1063	16	—	78	—	93	+	+
1064	—	31	120	55	140	+	0
Group 172. Diet 124 + carotene fraction of the non-saponifiable matter (corresponding to 6 % fat in the first 8 days, thereafter to 12 %):							
1065	—	30	170	55	1½	0	0
1066	—	29	185	50	4	0	0
1067	23	—	137	—	—	0	0
1068	—	31	118	55	4	0	0
1069	—	31	120	50	1½	0	0

Table I (*cont.*).

Chick No.	Age in days when: Dead	Age in days when: Killed	Weight g.	Haemoglobin (Sahli)	Time of clotting mins.	Haemorrhages: In the lining of the gizzard	Haemorrhages: Elsewhere		
FRACTIONS OF HOG-LIVER (*cont.*).									
Group 185. Diet 124 + 4 % hog-liver fat which had been heated on a boiling water-bath for 12 hours in contact with air (as a 1–2 mm. deep layer):									
1136	—	30	220	58	2	+	0		
1137	—	30	165	52	5½	0	0		
1138	—	30	200	50	6	?	0		
1139	—	30	220	55	1½	0	0		
1140	—	30	155	58	2	0	0		
ADDITIONS OF VITAMINS A, D AND E.									
Group 154. Diet 124 + (extra) 4 % cod-liver oil:									
968	—	27	140	29	>60	+	+ +		
969	—	27	112	32	>60	0	+		
970	—	13	84	52	>120	0	+ +		
971	—	24	125	50	>60	+	+ +		
972	—	24	105	40	>60	+	+ +		
Group 182. Diet 124 with halibut-liver oil instead of cod-liver oil (4 %) (1 g. of this oil contains 42,000 I.U. A and 2800 I.U. D):									
1121	—	27	135	35	>205	+	0		
1122	13	—	78	—	—	+	+ +		
1123	30	—	175	—	—	+	+ +		
1124	—	30	108	38	230	0	0		
1125	—	27	171	38	>230	+	0		
Group 175. Diet 124 + daily doses of "vogan" (1 ml. vogan contains 120,000 I.U. A):								Vogan Total ml.	I.U.
1084	—	33	164	42	18	+	0	4·25	510,000
1085	—	33	188	43	>240	0	0	4·25	510,000
1086	—	33	212	41	>300	+	+	4·25	510,000
1087	15	—	50	—	—	+	+ +	1·1	132,000
1088	—	33	211	40	>270	+	0	4·25	510,000
Group 178. Diet 124 + daily doses of carotene (Hoffmann La Roche):								Carotene Total mg.	
1099	—	31	232	42	7½	+	0	9·6	
1100	—	28	124	38	18	+	+ +	8·6	
1101	—	31	212	48	12	+	+	9·6	
1102	23	—	175	—	—	+	+	3·05	
1103	—	28	121	38	>120	0	+ +	8·6	
Group 163. Diet 124 + daily doses of "vigantol" (1 ml. vigantol contains 15,000 I.U. D):								Vigantol Total ml.	I.U.
1014	—	34	175	48	>120	+	+	0·21	3150
1015	—	28	152	30	>60	+	+	0·15	2250
1016	33	—	190	40	>60	+	+	0·20	3000
1017	—	34	200	52	>120	+	+	0·21	3150
1018	—	34	110	50	115	+	+	0·21	3150
Group 155. Diet 124 + 4 % wheat germ oil:									
973	—	34	218	50	60	+	+		
974	—	6	20	—	—	—	—		
975	—	42	375	50	60	0	0		
976	—	31	261	50	>60	+	+		
977	—	32	233	45	>60	0	0		
Group 148. Diet 60 + 10 % wheat germ oil:									
938	37	—	110	58	>30	+	+		
939	—	19	39	10	>60	+	0		
940	—	44	150	35	27	+	+		
941	—	44	120	35	35	+	+		
942	—	44	175	45	115	+	?		

Table I (*cont.*).

Chick No.	Age in days when		Weight g.	Haemo-globin (Sahli)	Time of clotting mins.	Haemorrhages	
	Dead	Killed				In the lining of the gizzard	Else-where
ADDITIONS OF VITAMINS A, D AND E (*cont.*).							
Group 186. Diet 111 + 24 % wheat germ oil:							
1141	—	30	143	58	18	+	0
1142	15	—	50	—	1	0	0
1143	—	30	170	51	1½	+	0
1144	—	30	200	52	12	+	0
1145	—	30	160	54	2	+	0
DIFFERENT FATS AND OILS.							
Group 110. Diet 60 + 10 % butter fat:							
746	—	31	80	<8	90	+	0
747	—	38	138	45	7	0	0
748	—	38	144	31	11	+	+
749	21	—	64	—	—	+	+
750	—	21	72	50	>60	?	0
Group 128. Diet 60 + 10 % cod-liver oil:							
842	—	20	47	20	>120	+	+
843	17	—	38	—	—	+	+
844	—	20	51	26	>60	+	+
845	—	18	70	31	>30	+	+
846	—	13	38	10	>10	+	0
Group 130. Diet 60 + 10 % soya bean oil:							
852	—	27	112	50	5	+	+
853	—	23	113	58	7	?	+
854	—	42	168	53	5	+	0
855	23	—	73	—	—	0	?
856	—	42	191	55	10	0	0
Group 131. Diet 60 + 10 % copra oil:							
857	—	13	41	<10	>30	+	0
858	—	23	74	44	120	?	0
859	12	—	37	—	—	+	0
860	—	23	97	50	27	0	0
861	—	23	82	50	5	?	0
Group 140. Diet 60 + 10 % hog-liver fat:							
902	—	28	45	30	2½	0	0
903	—	28	67	56	1	0	0
904	—	24	39	50	2½	?	0
905	—	28	89	54	2	0	0
906	—	28	68	52	4	0	0
Group 141. Diet 60 + 10 % rape oil:							
907	—	37	178	50	28	0	0
908	—	37	140	53	2½	0	0
909	29	—	50	—	—	?	+
910	—	36	130	38	>14	?	+ +
911	—	37	154	55	95	0	0
Group 142. Diet 60 + 10 % linseed oil:							
912	—	28	55	18	20	+	0
913	—	28	83	25	30	+	0
914	—	24	49	<10	>60	+	+ +
915	—	28	89	47	—	+	+
916	—	26	58	<5	>70	+	0
Group 191. Diet 124 + 10 % lard:							
1169	—	30	199	55	24	+	0
1170	—	30	153	47	>110	+	0
1171	—	30	149	48	22	?	0
1172	9	—	64	—	—	+	+ +
1173	—	30	149	42	>150	+	0

Table I (*cont.*).

Chick No.	Age in days when		Weight g.	Haemoglobin (Sahli)	Time of clotting mins.	Haemorrhages	
	Dead	Killed				In the lining of the gizzard	Elsewhere
HEN'S EGG.							
Group 54. Diet 49* + 10 % fresh egg-yolk:							
431	—	49	241	—	—	+	+ +
432	—	52	252	—	—	0	+ +
433	—	20	64	—	—	+	+
434	—	55	452	64	—	?	0
435	—	55	352	47	—	0	+ +
Group 55. Diet 49 + 10 % fresh egg-white:							
436	—	18	43	—	—	+	+
437	—	18	42	—	—	+	0
438	—	18	54	—	—	+	+ +
439	—	18	53	—	—	+	+ +
440	18	—	57	—	—	?	+
Group 56. Diet 49 in which 20 % caseinogen was replaced by 20 % commerical desiccated egg-white:							
441	—	39	86	—	—	+	0
442	—	27	66	—	—	+	0
443	—	39	73	—	—	+	0
444	—	24	53	—	—	+	0
445	—	31	45	—	—	+	?
Group 167. Diet 60 in which 25 % sucrose was replaced by 25 % commerical desiccated egg-yolk:							
1034	—	38	270	52	½ - 4½	0	0
1035	—	38	220	50	8½–28	0	0
1036	—	39	295	45	4	?	0
1037	—	38	300	50	8½–28	0	0
1038	–	38	322	55	13½	0	0
1082	—	33	322	55	7	0	0

* Dam and Schönheyder [1934].

RESULTS AND DISCUSSION.

Table I shows clearly that certain cereals and seeds will protect against the disease to a large extent whilst others are practically ineffective. Hemp seed appears to be particularly protective whilst unpolished rice, sunflower seed, yellow corn and rye are nearly valueless. The other cereals which have been tested appear to occupy a middle position.

Certain vegetables such as tomatoes, kale, orange peel *etc.* appear to have a fairly good action against the disease.

Among the organs so far tested, hog-liver is the most potent source of the active principle, while calf-brain, ox-lung and muscle are extremely poor. Ox-kidney, adrenals and calf-thymus occupy a middle position.

The hen's egg does not appear to be a very rich source, but the yolk will no doubt afford protection when given in large quantities.

All symptoms—clotting time, haemorrhages in the gizzard and other organs and haemoglobin content—are taken into consideration in estimating the condition of the animals, since at the present state of the investigation, the possibility that certain factors in the food may render the gizzard more resistant or may counteract the tendency to haemorrhage which is in turn due to the retarded clotting, has not been excluded. It is also possible that other factors or properties of the food may reduce the resistance of the gizzard or favour the occurrence of spontaneous haemorrhages.[1] Extremely retarded growth will undoubtedly counteract spontaneous haemorrhages.

It is not probable that the low haemoglobin values which are found in animals with large haemorrhages have anything to do with lack of copper, since similar results are obtained when the diet contains 10 % marmite. 1 g. marmite has been found to contain about 0·06 mg. Cu.

The results from groups 105, 124, 139 and 140 clearly demonstrate that the active principle in hog-liver fat can be extracted with ether. Corresponding experiments with hemp seed—groups 97, 120 and 132—lead to the conclusion that the ether extract of hemp seed is protective and that the action of the extracted seed is diminished but not entirely lost, thus suggesting that the active principle of hemp seed—like the carotene of green leaves—is not altogether in association with the fat. A series of other fats and oils was tested but none of them was found to be very potent.

McFarlane *et al.* [1931] have made the observation that chicks reared on an artificial diet with ether-extracted meat meal or ether-extracted white fish meal as the source of protein suffered from a tendency to haemorrhages whilst this was not the case when the meat meal or fish meal was not extracted with ether. This is in line with the results of the present paper. McFarlane *et al.* reported, however, that they could not observe haemorrhages when caseinogen, extracted or non-extracted, was used as protein. This latter statement is incompatible with the findings of Dam [1929; 1930] and Dam and Schönheyder [1934] as well as with the present experiments in which the diets contain caseinogen.

Fractionation of hog-liver fat into fatty acids—group 125—and non-saponifiable matter—group 126—revealed the fact that the antihaemorrhagic factor is to be found in the non-saponifiable fraction, and by further fractionation—groups 150, 151 and 152—the factor was found in the easily soluble non-sterol fraction, the sterols and a fraction sparingly soluble in ether being inactive. From a solution in light petroleum the factor could not be removed by repeated shaking with 90 % methyl alcohol—groups 171 and 172. In this respect the antihaemorrhagic factor behaves like vitamin E [Olcott and Mattill, 1931] and differs from vitamin A [Wolff *et al.*, 1930]. (The paper of the last-mentioned authors deals with the partition of vitamin A between light petroleum and ethyl alcohol, but, according to a personal communication from Prof. Wolff, it has been established that methyl alcohol behaves in the same manner.)

Addition of very large amounts of vitamins A and D to the diet does not prevent the disease—group 154, extra 4 % cod-liver oil—group 182, replacement of the cod-liver oil of the basal diet by halibut-liver oil containing 42,000 I.U. of A and 2800 I.U. of D per g. whereby the amounts of vitamins A and D are raised materially—group 175, addition of the concentrated vitamin A preparation "vogan"—group 163, addition of pure vitamin D in sesame oil "vigantol".

Commercial carotene (Hoffmann La Roche), which according to the statement of the firm contains 70–80 % β- and 30–20 % α- *plus* traces of γ-carotene, does not prevent the disease when given in quantities up to 9 mg. in 1 month.

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[1] Certain food components, such as desiccated hog-liver, hog-liver fat, large amounts of cod-liver oil may produce symptoms resembling polyneuritis (3 animals in group 105, 3 in group 125, 2 in group 139). This polyneuritic condition is often accompanied by multiple minute haemorrhages in the cerebellum. Such haemorrhages, however, appear to be independent of the clotting time and general haemorrhagic condition since they may appear in groups where clotting time is normal and when no haemorrhages occur in other organs. The origin of these haemorrhages and of the polyneuritic symptoms is being studied further. Polyneuritic chicks are more liable to traumatic haemorrhages than others, owing to their convulsive movements.

According to Brockman and Völker [1934] hemp seed, which is very active, is practically devoid of carotene. The conclusion is therefore justified that the antihaemorrhagic vitamin cannot be identical with vitamins A or D or with α- or β-carotene. The antihaemorrhagic vitamin bears some resemblance to vitamin E. An experiment—group 185—in which the liver fat had been heated in a boiling water-bath for 12 hours in a 1–2 mm. thick film, showed that the factor is not destroyed to any great extent under these circumstances. According to Evans [1932] vitamin E will resist such treatment. On partition between light petroleum and 90 % methyl alcohol the vitamin behaves, as mentioned, like vitamin E. It appears that a loss occurs during the saponification.

Freshly prepared wheat germ oil, however, did not protect when given at a level of 4 or 10 % in the diet, and 24 % yielded an incomplete protection—groups 155, 148 and 186. 62 % wheat germ was rather inactive—group 86. Owing to the fact that such large amounts of wheat germ oil are insufficient to prevent the disease while 3–4 % of hog-liver fat is entirely protective, it must be considered very unlikely that the antihaemorrhagic vitamin is identical with vitamin E.

It is therefore suggested that the term *vitamin K* be used for the antihaemorrhagic factor until its chemical nature has been elucidated.

Comparison of the weights of the animals in groups 105, 124 and 172 appears to indicate that vitamin K does not promote growth.

The question whether the lack of this vitamin will produce the same symptoms in other animals than chicks is under investigation.

SUMMARY.

1. The nature and distribution of the antihaemorrhagic vitamin of the chick have been investigated.

2. The vitamin is fat-soluble and one of its richest sources is hog-liver fat, whilst cod-liver oil is practically devoid of it. The hen's egg contains the vitamin in the yolk but is apparently not an extremely rich source. In the vegetable kingdom hemp seed and certain vegetables are good sources, yellow corn, unpolished rice and sunflower seeds are very poor.

3. The vitamin occurs in the easily soluble non-sterol fraction of the unsaponifiable matter.

4. It cannot be identical with vitamin A or D because large amounts of these vitamins in the diet are completely ineffective in preventing the disease. It bears some resemblance to vitamin E with respect to solubility and resistance to heating in air, but is held to be different from E because large quantities of wheat germ and wheat germ oil do not afford complete protection against the disease.

5. It is suggested that the term *vitamin K* be used for the antihaemorrhagic factor.

6. The further chemical nature and the possible importance of the vitamin for other animals than the chick are under investigation.

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REFERENCES.

Brockmann and Völker (1934). *Z. physiol. Chem.* **224**, 193.
Dam (1929). *Biochem. Z.* **215**, 475.
—— (1930). *Biochem. Z.* **220**, 258.
—— and Schönheyder (1934). *Biochem. J.* **28**, 1355.
Evans (1932). *J. Amer. Med. Assoc.* **99**, 469.
McFarlane, Graham and Richardson (1931). *Biochem. J.* **25**, 358.
Olcott and Mattill (1931). *J. Biol. Chem.* **93**, 61.
Schönheimer and Dam (1933). *Z. physiol. Chem.* **215**, 59.
Schönheyder (1935). *Nature*, **135**, 653.
Wolff, Overhoff and van Eckelen (1930). *Deutsch. med. Woch.* **56**, 1428.

CLV. GROWTH OF BLOW-FLY LARVAE ON BLOOD AND SERUM.

II. GROWTH IN ASSOCIATION WITH BACTERIA.

By RALPH PERCIVAL HOBSON.

From the Department of Entomology, London School of Hygiene and Tropical Medicine.

(*Received April 11th, 1935.*)

In the previous paper of this series [Hobson, 1933] it was shown that aseptic blow-fly larvae cannot develop on sterile blood or serum owing to lack of vitamin "B". The present paper deals with the growth of larvae on these foods when bacteria are present. The species of blow-fly used was *Lucilia sericata*, Meig., the Sheep Maggot Fly. The nutrition of these larvae on blood and serum has a direct bearing on the sheep maggot problem. Mackerras and Freney [1933] examined infested sheep in Australia and found that the maggots feed at first on a serous exudate and later on blood; this has been confirmed by field observations made by the present author in North Wales[1].

METHODS.

The larvae were reared on sterile horse blood and serum. The procedure adopted in the growth trials with pure bacterial cultures was identical with that used in the aseptic experiments [Hobson, 1933], except that the food was inoculated with the organism to be tested. The method of rearing naturally infected larvae on blood was as follows: 0·6 ml. samples of blood were diluted to 1·1 ml. and absorbed on standard lengths of surgical bandage in small test-tubes. A batch of eggs laid by one fly was incubated on a small piece of beef until the weight per larva was approximately 0·5 mg. 3 larvae were transferred to each tube, which was then closed with fine muslin and incubated in a moist atmosphere at 27°. Growth was measured by weighing the larvae at intervals. If eggs were used instead of young larvae, the results were essentially the same, but the individual variation was greater. In parallel experiments good agreement was found between duplicates, but in different series the growth rate varied somewhat, perhaps owing to differences in the bacterial flora.

Growth of infected larvae on blood and serum.

Although *Lucilia* larvae cannot develop on blood under aseptic conditions, fair growth occurs when bacteria are present owing to synthesis of vitamin "B". Fig. 1 shows some typical results. It will be seen that infected larvae grew more slowly on blood than on muscle (their natural food), and that the difference almost disappeared when yeast autolysate was added to blood. However, the effect of this extract was not entirely due to organic substances since yeast ash stimulated growth on infected blood. These curves also show that a considerable time elapsed before the larvae began to make rapid growth on blood; thus, when

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[1] Most of the work described in this paper was carried out in London. The writer is now investigating the sheep maggot problem at the School of Agriculture, University College of North Wales, Bangor.

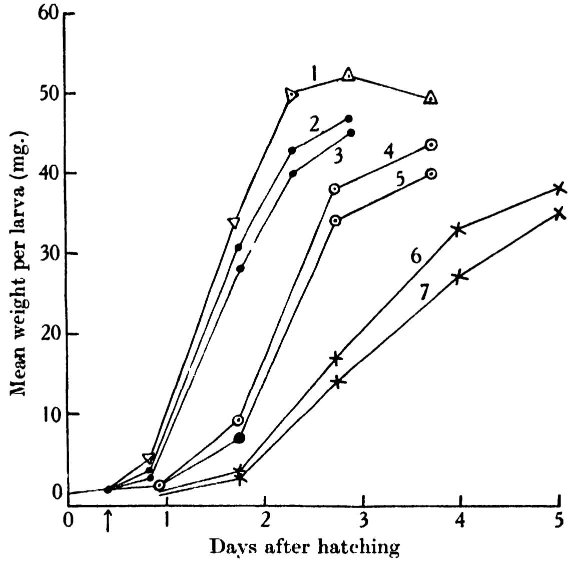

Fig. 1. Growth of blow-fly larvae on meat and blood. 1 △ meat; 2 and 3 • blood + yeast autolysate; 4 and 5 ⊙ blood + yeast ash; 6 and 7 × blood alone; ↑ indicates transfer of larvae from meat to blood.

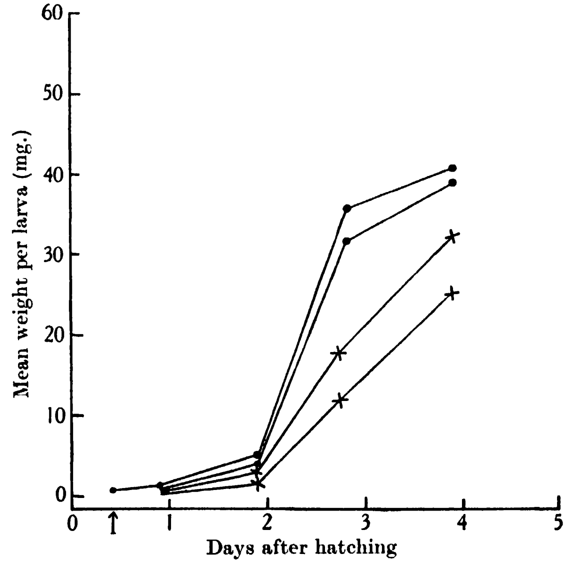

Fig. 2. Growth of blow-fly larvae on blood. • coagulated; × fluid. ↑ indicates transfer of larvae from meat to blood.

yeast ash was present, the larvae developed very slowly during the first day, growth later becoming almost normal. The period of slow growth was lengthened if the larvae were transferred each day to sterile blood. When aseptic larvae are fed on a diet which contains an insufficient amount of vitamin "B", growth is at first normal and later falls off, the reverse of what was found with infected larvae. In larvae reared without sterile precautions, the alimentary tract contains large numbers of bacteria; if this were the site of vitamin synthesis, a period of delayed growth would not follow the transference of larvae to a deficient diet. These results, therefore, suggest that the synthesis of vitamin "B" occurs in the food outside the body and not in the gut. This was confirmed by the observation that a change in the physical state of the food, heat-coagulation of the blood, resulted in improved growth (Fig. 2). This effect was not due to a difference in the bacterial flora as it will be shown later that coagulation of the blood has the same effect when pure cultures are used. Since the conditions in the gut are much the same whether the blood is fluid or coagulated, this result must be due to a difference in the growth of bacteria in the medium.

Growth-promoting effect of yeast ash.

Both muscle and yeast are rich in potassium and phosphorus, but blood contains relatively small amounts of these elements. Growth tests were, therefore, made with blood enriched with potash and phosphate; various salts were used, the amount being equivalent to 0·5 % sodium chloride. Before being mixed with blood, the salt solutions were neutralised with sodium hydroxide.

Table I. *Effect of mineral salts on the growth of larvae on blood.*

Substance added	Weight of larvae in mg. after two days' growth
1·1 % KH_2PO_4	34, 31
0·6 % KCl	10, 12
1·0 % NaH_2PO_4	35, 33
0·5 % NaCl	15, 9
1 % yeast ash	40, 34
2 % yeast ash	36, 33
Nil	12, 16

Table II. *Effect of mineral salts on the growth of larvae on serum.*

Substance added	Weight of larvae in mg.	
	3rd day	4th day
1·1 % KH_2PO_4	41, 46	—
1·0 % NaH_2PO_4	14, 16	25, 27
0·6 % KCl	—	4, 7
Nil	—	4, 5

The results given in Tables I and II show that the growth-promoting effect of yeast ash was entirely due to phosphate on a blood diet; in the case of serum, potash also improved growth. There appear to be two possible explanations of these results: (1) the amounts of phosphorus and potassium in blood and serum are insufficient for the requirements of the larvae; (2) the addition of potassium phosphate stimulates bacterial growth and synthesis of vitamin "B". Phosphorus analyses were, therefore, made on larvae and on the sample of blood used in these experiments. Organic matter was destroyed by oxidation with sulphuric and nitric acids, phosphorus being estimated colorimetrically by the Warren and Pugh [1930] modification of the method of Denigès [1920]. The results and calculations which follow are all expressed on a dry weight basis.

Table III shows the results of the phosphorus determinations. It was also necessary to know what proportion of blood is converted into larval tissue. An

Table III. *Phosphorus content of blow-fly larvae.*

	% P on dry weight
Larvae reared on meat	0·76
Larvae reared on meat	0·70
Larvae reared on blood	0·77
Blood sample	0·125

excessive number of larvae were, therefore, fed on a mixture of blood and sodium phosphate until the food was entirely consumed. 32 % of the original dry weight of the blood was recovered as dried larvae. Thus, 100 g. of the blood supplied enough food, other than phosphorus, for the formation of at least 32 g. of larval tissue, but only contained as much phosphorus as 16 g. of larvae. Blood is, therefore, deficient in phosphorus, supplying about half the amount required by *Lucilia* larvae for normal growth. This calculation agrees well with the results of growth tests carried out with different amounts of added phosphate.

Table IV. *Effect of phosphate on growth of larvae on blood.*

mg. PO_4 added per 1 ml. blood	% P in blood (dry weight)	Weight of larvae in mg. after 2 days' growth
0	0·125	12, 14
0·4	0·19	19, 23
0·8	0·26	29, 30
1·6	0·39	34, 35
3·2	0·66	33, 31

Table IV shows that doubling the amount of phosphorus in blood produced optimum growth. It is of interest to note that the phosphorus content of larvae does not fall on a diet of blood alone; presumably they concentrate this element by oxidising a larger proportion of their food than usual. Although blood is poor in phosphorus as compared with muscle, this result is surprising as many insects feed solely on blood throughout their whole life cycle. However, blow-fly larvae grow very rapidly and convert a high percentage of their food into tissue. Blood-sucking insects probably burn a larger proportion of their food, so obtaining more mineral matter per unit weight of tissue formed. It should be noted that the various basal diets used in the aseptic experiments on vitamin "B" [Hobson, 1933] all contained yeast fractions; these diets were not deficient in mineral salts as larvae grew well on them when bacteria were present.

Growth of larvae on blood infected with pure cultures.

In order to determine whether synthesis of vitamin "B" is due to a specific organism, sterile larvae were fed on blood infected with pure cultures of various organisms isolated from blow-fly larvae. The numbers assigned to the different types are the same as those used in the description of the bacterial flora [Hobson, 1932]; these are non-lactose-fermenting bacilli, which predominate in the gut of the larvae and in blown meat. Tests were also made with *B. coli*. The basal diet consisted of sterile blood mixed with an equal volume of 0·5 % sodium phosphate solution. The food was prepared with aseptic precautions; to ensure sterility, the tubes containing the blood were heated, either for 40 minutes at 75°, when coagulated blood was required, or twice at 50° for 30 minutes. The results (Table V) show the weights of the larvae after 3–4 days' growth. Each figure represents the mean weight of the larvae in one tube. It will be seen that with coagulated blood all the organisms tested produced good growth, the larvae

Table V. *Growth of larvae on blood infected with pure cultures. 1 °/₀ sodium phosphate added to blood.*

Organism used	Weight of 3-day old larvae (mg.)	
	Coagulated blood	Fluid blood
(1) Bacillus "A"	18, 22	2, 2·5
(4) "	34, 35	3, 6
(5) *Proteus vulgaris*	31, 35	—
(6) "	42, 44	16, 23
B. coli	38, 45	5, 6
Mixed infection; unsterilised eggs used	39, 43	30, 34
Aseptic	0·5, 1	—

developing at about the same rate as when the infection was mixed. However, the same organisms gave very poor results when the blood was not coagulated, the growth rate being distinctly inferior to that of unsterilised larvae. An attempt was made to isolate organisms able to promote larval growth on fluid blood. Single colonies were isolated from agar plates inoculated with blood on which larvae were feeding. One of these, No. 6, gave fair growth on uncoagulated blood.

These results show that, when larvae are growing on a deficient diet, synthesis of vitamin "B" is not due to a specific organism, but to various bacilli which develop in the gut and in the food. It was of interest to find that larvae grew well on coagulated blood when infected with *B. coli*. Since aseptic larvae respond to vitamin B_1 (Peters's antineuritic concentrate), when reared on sterile blood mixed with autoclaved yeast, this shows that *B. coli* can synthesise vitamin B_1 under the experimental conditions used. Previous workers have obtained conflicting results as to the ability of *B. coli* to synthesise this vitamin [see Peskett, 1933]. The marked effect of coagulating the blood appears puzzling at first sight. The explanation may be that bacteria would be concentrated on the surface of the blood coagulum. The larvae in this case feed by scraping at the surface, and would ingest a larger proportion of bacteria. Similarly; the stimulating effects of the larvae on bacterial growth, mechanical dispersion and excretion of trypsin [Hobson, 1931], would be more effective when larvae are feeding on the surface of a solid. Growth on a diet lacking in vitamin "B" is an autocatalytic reaction; bacterial action promotes larval growth, which, in turn, stimulates bacterial growth. Hence, the effect of any favourable factor tends to become exaggerated.

SUMMARY.

1. Blow-fly larvae are able to grow on blood when bacteria are present. Since aseptic larvae fail to develop on blood unless vitamin "B" is added, the natural flora must supply this vitamin.

2. The site of vitamin synthesis appears to be the food and not the intestine.

3. Blood contains insufficient amounts of phosphorus for the normal growth of larvae. Serum is deficient in both phosphorus and potassium.

4. When phosphate is added, larvae develop on unsterile blood at the usual rate except for a lag in the early stages.

5. Larvae can grow readily on blood infected with pure cultures of various bacilli isolated from the intestine and from blown meat. *B. coli* proved equally effective.

I wish to record my thanks to the Agricultural Research Council for a grant which entirely financed this work.

REFERENCES.

Denigès (1920). *Compt. Rend. Acad. Sci.* **171**, 802.
Hobson (1931). *J. Exp. Biol.* **8**, 109.
—— (1932). *J. Exp. Biol.* **9**, 128.
—— (1933). *Biochem. J.* **27**, 1899.
Mackerras and Freney (1933). *J. Exp. Biol.* **10**, 237.
Peskett (1933). *Biol. Rev.* **8**, 1.
Warren and Pugh (1930). *J. Agric. Sci.* **20**, 532.

CLVI. ON A FAT-SOLUBLE GROWTH FACTOR REQUIRED BY BLOW-FLY LARVAE.

I. DISTRIBUTION AND PROPERTIES.

By RALPH PERCIVAL HOBSON[1].

From the Department of Entomology,
London School of Hygiene and Tropical Medicine.

(*Received April 11th, 1935.*)

Although there is good evidence that insects require vitamin B [see Uvarov, 1928; Hobson, 1933], little is known of their need for fat-soluble growth factors. The previous work will not be reviewed in detail at this stage, except to say that the suggestions of some workers that insects require vitamin A or E have little foundation. The present work arose from the observation that filtration of peptic digests of meat removes a substance which is essential for the growth of blow-fly larvae (*Lucilia sericata* Mg.) and which occurs in certain fats. An investigation was therefore made of the distribution and properties of the active substance.

Methods.

The basal diet used in this work was prepared as follows. Lean beef was digested with pepsin for 4 days at p_H 2, the digest being filtered through filter-paper and the solution concentrated on a water-bath. After a small amount of aqueous yeast extract had been added and the reaction adjusted to p_H 6 with

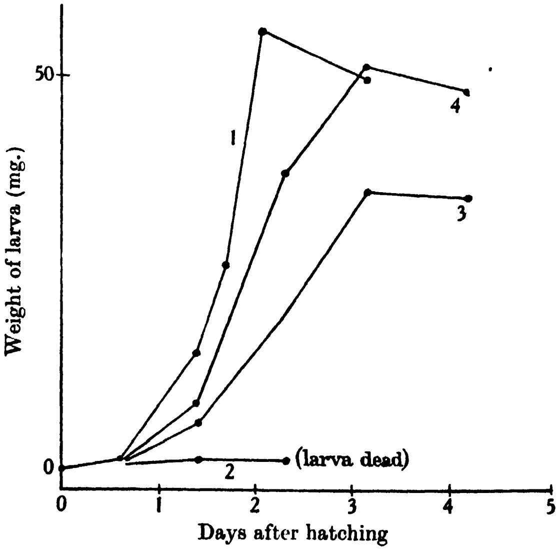

Fig. 1. Growth of blow-fly larvae on meat and peptone. 1, Meat; 2, peptone; 3, peptone + 2 mg. cod-liver oil; 4, peptone + 8 mg. cod-liver oil.

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[1] Now at the School of Agriculture, University College of North Wales, Bangor.

sodium hydroxide, the mixture was diluted with water to make the concentration of peptone 15 % (based on a dry weight estimation made before the addition of yeast extract and alkali). This solution was stored in a refrigerator with chloroform as an antiseptic; before use samples were heated to remove the chloroform. Attempts to rear larvae on oil emulsions gave unsatisfactory results. Since the peptone solution could not be properly mixed with fat, one larva was used in each test. 0·3 ml. of peptone solution was absorbed on 60 mg. of cotton wool, pressed into a pellet, in a small test-tube. The oil was measured out with a platinum loop and smeared over the cotton wool; under standardised conditions, the loop delivered approximately 2 mg. of oil. One egg was placed in each tube, which was closed with fine muslin gauze, and the larvae were reared at 27° in a moist incubator. The amount of peptone supplied was equivalent to 180 mg. of fresh muscle; this provided sufficient food since a larva can grow to the full size (50 mg.) on 90 mg. of muscle.

Fig. 1 shows the growth of larvae on peptone and on muscle. On peptone alone, larvae fail to grow and usually die on the second day; however, they remain alive under aseptic conditions for 5 or 6 days without growing. When an active oil is given in excess, growth is almost normal and the larva reaches the full size in 70–80 hours; with an insufficient amount of the essential factor, growth is slower and the final weight less. Fig. 1 includes some results obtained with cod-liver oil. Since the rate of growth was not usually so uniform as this, the weight on the fourth day was taken as the criterion of growth.

Table 1. *The effect of various substances on the growth of blow-fly larvae on peptone diet.*

Substance	Dose (mg.)	Weights of larvae (mg.) on 4th day	
Cod-liver oil	8·0	43, 46, 47, 52	
	4·0	30, 32, 42, 43	
	2·0	16, 34, 38	
	1·0	12, 21, 27	
Butter	8·0	27, 37, 42, 51	
	4·0	21, 25, 36	
	2·0	12, 17, 25	
Beef muscle oil	0·8	47, 49, 51	
	0·4	32, 47, 48	
	0·2	17, 25, 35	
Wheat germ oil	4·0	48, 49, 51	
	2·0	37, 40, 43, 46	
	1·0	33, 39, 40	
	0·5	24, 30, 36	
Wool wax	0·5	45, 49	
	0·25	45, 47	
	0·12	33, 47, 48	
	0·06	16, 25, 35	
Egg yolk	15·0	50, 53	
	7·5	44, 48	
	3·7	43, 49	
	1·9	28, 35	
Ovolecithin	3·0	47, 50	
	1·5	42, 46	
	0·7	29, 36	
Lard	8·0	4, 5, 7, 11	Weights on 3rd day Larvae usually died before 4th day
Olive oil	8·0	3, 4, 5, 5, 7	
Nil	—	Larvae died on 2nd or 3rd day, weight about 1 mg.	

Table II. *Distribution of the growth factor.*

Substance	Potency	Active dose mg.
Wool wax	+ + +	0·1
Beef muscle oil	+ + +	0·4
Ovolecithin (technical)	+	2
Wheat germ oil	+	3
Egg yolk	+	4
Cod-liver oil	+	6
Butter	+	8
Lard	−	>8
Olive oil	−	>8

Active dose = smallest amount required for normal growth of 1 larva on 0·3 ml. of peptone solution.

Effect of various fats and oils on growth.

In these experiments the fat-soluble substances were diluted with an inactive oil, olive oil, and tested at different concentrations, each larva receiving 8 mg. of oil. Tests were also made with egg yolk and commercial ovolecithin mixed with the peptone solution as suspensions. The mortality in these trials was high, especially with certain oils and at low concentrations; when the larva was dead or moribund on the fourth day, the experiment was repeated. The results of the successful tests are collected in Table I and summarised in Table II. The active dose was taken to be the smallest amount which produced a larva weighing more than 45 mg. on the fourth day from hatching.

Reference to Table II shows that cod-liver oil and butter fat proved relatively poor sources of the growth factor; wool wax and muscle oil were the most active of the substances tested, wheat germ oil and ovolecithin giving intermediate values. Lard and olive oil were almost inactive. These results show clearly that the active substance is not vitamin A, D or E. Vitamins A and D are present in large amounts in cod-liver oil and butter; also they are absent from wool wax [Drummond and Baker, 1929], the richest source of the larval factor. Since wheat germ oil proved less potent than muscle oil and wool grease, the larval factor is not vitamin E. It should be noted that the muscle oil was obtained from a sample of beef which yielded only 1·3 % of ether-soluble material, the extract being very rich in unsaponifiable matter.

Chemical properties of the growth factor.

In order to test the stability of the factor to oxidation, growth tests were carried out with cod-liver oil, which had been aerated for 24 hours at 90–95°. This product appeared to be toxic as a large proportion of the larvae died. However, since some grew tolerably well, it is concluded that the active substance is resistant to oxidation. It is also stable to alkaline hydrolysis and can be recovered quantitatively in the unsaponifiable residue. Lecithin was used for chemical fractionation since it is rich in the blow-fly factor; it also stimulates the growth of *Drosophila* on a synthetic peptone diet [Guyenot, 1917]. 40 g. of technical ovolecithin, hydrolysed with alcoholic potash on a boiling water-bath for 45 minutes, yielded 1·4 g. of unsaponifiable material. This was fractionated by recrystallisation from alcohol into colourless crystals of cholesterol and a dark-coloured gummy material which consisted largely of cholesterol. Growth tests with olive oil solutions showed that the unsaponifiable residue contained the whole of the original potency. The active dose, for 1 larva on 0·3 ml. of peptone solution, was 30–60γ, equivalent to 1–2 mg. lecithin; the minimum amount of lecithin which produced good growth was about 2 mg. (when emulsified in the same volume of peptone solution). Recrystallisation of the unsaponifiable residue from alcohol did not concentrate the activity, since both fractions proved equally potent. In order to determine whether the active substance is cholesterol itself or an impurity, further growth trials are being made with sterol samples from different sources.

DISCUSSION.

The results of the present experiments show that blow-fly larvae require a fat-soluble factor which is present in the unsaponifiable residue. This substance not only stimulates growth, but seems to possess, also, an "anti-infective" function. Under normal conditions *Lucilia* larvae are highly resistant to bacterial infection and grow readily on meat however badly decomposed. This immunity extends also to diets which are deficient in vitamin B and mineral salts; thus, although larvae grow extremely slowly on infected serum, they show no other ill effects. With the peptone diet alone, death always occurred after two or three days; also, larvae receiving small doses of the fat-soluble factor often died before the end of the experiment. The cause of death was excessive bacterial action since the mortality was reduced by sterile precautions and was not found in aseptic cultures. The fat-soluble factor seems, therefore, to play a part in the immunity of these larvae to infection.

The distribution of the growth factor shows clearly that it is not vitamin A, D or E; thus, wool grease proved more than ten times as potent as cod-liver oil or wheat germ oil. However, the results obtained with blow-fly larvae agree with the findings of previous workers on insects. Guyenot [1917] showed that *Drosophila* larvae require a growth factor which is supplied by yeast fat or egg lecithin, but not by triolein. Bacot and Harden [1922], when studying the response of this insect to vitamin B, found it necessary to include butter in the basal diet. Richardson [1926] showed that *Ephestia* larvae could not grow on wheat flour which had been extracted with fat solvents; wheat oil and egg yolk restored growth, olive oil and lard being ineffective. Although butter gave poor results, Richardson suggested that the active substance might be vitamin A. Moskalenko [1933] concluded that cockroaches require vitamin E and possibly other fat-soluble growth factors. Using an artificial diet, he found wheat-germ oil the most effective in stimulating growth; fish oil (?liver oil)[1] was also active, butter producing slow growth. Zabinski [1926; 1928], working with the same insect, obtained inconclusive results. Michelbacher *et al.* [1932] found that blow-fly larvae could not develop aseptically on a synthetic diet unless cod-liver oil or butter was included. Table III summarises the responses of various insects to fat-soluble growth factors.

The fat-soluble vitamin requirements of higher animals do not apparently differ in the same way as do their needs for water-soluble vitamins. It may be assumed, therefore, in the absence of evidence to the contrary, that the fat-soluble growth factors required by insects do not vary among different types. It will be seen from Table III that the results obtained with different insects bear a general resemblance. Since the blow-fly factor is not vitamin A, D or E, it is inferred that insects do not require the same fat-soluble growth factors as higher animals. Furthermore, the present results suggest a relationship between the blow-fly factor and sterol. Thus, the only substance which compared in activity

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[1] I was unable to see the original paper, but was fortunate in borrowing an English translation. The fish oil was probably a liver oil since Moskalenko refers to it as well known to be rich in vitamin A.

Table III. *The response of insects to fat-soluble growth factors.*

Insect	*Lucilia*		*Blatta*	*Ephestia*	*Drosophila*	
Investigator ...	Hobson	Michelbacher *et al.*	Moskalenko	Richardson	Bacot and Harden	Guyenot
Wool wax	5	—	—	—	—	—
Wheat germ oil	4	—	3	2	—	—
Egg yolk	3	—	—	2	—	+
Cod-liver oil	2	+	2	—	—	—
Butter fat	1	+	1	1	+	—
Lard	0	0	—	0	—	—
Olive oil	0	—	—	0	—	—
Triolein	0	—	—	—	—	0

1, 2, 3 *etc.* (in same vertical column): order of increasing activity.
0 inactive.
+ active (no indication of potency).

with wool wax (mainly composed of sterol esters) was a sample of beef oil (Table II), which was undoubtedly rich in sterol. The other substances tested were relatively poor both in sterol and in the growth factor. Also, a sample of cholesterol obtained from ovolecithin proved highly active. The nature of the growth factor will be considered more fully in a later paper giving the results of tests with sterols from various sources.

SUMMARY.

1. Muscle oil contains a substance which is essential for the growth of blow-fly larvae. Other sources of the growth factor are, in order of decreasing potency: wool wax, wheat germ oil, egg yolk, cod-liver oil, butter. Olive oil and lard have little or no effect on growth.

2. These results agree with the observations of previous workers on insects, but the distribution of the blow-fly factor shows that it is not vitamin A, D or E.

3. The growth-promoting effects of various substances appear to run parallel with their sterol contents.

4. The active substance is present in the unsaponifiable residue after hydrolysis.

5. There was some evidence that the growth factor possesses also an "anti-infective" function.

I wish to record my thanks to the Agricultural Research Council for a grant which entirely financed this work.

REFERENCES.

Bacot and Harden (1922). *Biochem. J.* **16**, 148.
Drummond and Baker (1929). *J. Soc. Chem. Ind.* **48**, 232 T.
Guyenot (1917). *Bull. Biol. Fr. Belg.* **51**, 229.
Hobson (1933). *Biochem. J.* **27**, 1899.
Michelbacher, Hoskins and Herms (1932). *J. Exp. Zool.* **64**, 109.
Moskalenko (1933). *Nutr. U.S.S.R.* **2**, 28.
Richardson (1926). *J. Agric. Res.* **32**, 895.
Uvarov (1928). *Trans. Ent. Soc. London*, 255.
Zabinski (1926). *Compt. Rend. Soc. Biol.* **94**, 545.
—— (1928). *Compt. Rend. Soc. Biol.* **98**, 73, 78.

CLVII. THE LIBERATION OF METHYL SULPHIDE BY SEAWEED.

By PAUL HAAS.

From the Botanical Department of University College, London.

(*Received April 12th, 1935.*)

THE smell given off from seaweed is of two kinds—that associated with decomposing weed and that evolved by the weed in its natural condition. It is the smell of decomposing weed which is usually the more noticeable by the seaside, being characterised as a rule by a more or less distinct odour of hydrogen sulphide which arises in part, presumably, from the putrefactive reduction of the organically combined sulphates which form a marked characteristic of so many marine algae [Haas, 1921; Haas and Russell-Wells, 1923]. There do not appear to be any recorded observations on the nature of volatile odoriferous principles given off by undecomposed seaweed, and it is with this aspect of the question that the present communication deals. The immediate incentive for this investigation was the peculiarly penetrating, somewhat sickly odour, suggestive of a volatile phosphorus compound, given off from *Polysiphonia fastigiata* on drying.

The persistent occurrence of this smell, no matter from what locality or at what season of the year the material had been collected, suggested that the smell was due to some change inherent in the weed, associated either with the drying process or with injury due to cutting, and not to accidental contamination or to bacterial action; the latter suggestion was moreover rendered improbable by the short interval elapsing between the time of collection of the weed and the appearance of the smell. The fact that material which had been killed by immersion in boiling water no longer gave the smell suggested that the production of the smell was in some way connected with a disturbance of the normal metabolism of the plant. The investigation of this matter is reserved for a future occasion.

EXPERIMENTAL.

With a view to identify the odoriferous principle concerned a five-litre flask was filled as rapidly as possible with freshly gathered cut up weed. The flask, fitted with a cork through which passed a long tube reaching to the bottom and a short delivery tube, was placed in a large water-bath kept at 30°. By means of a filter-pump a current of air charged with the evolved gas was then drawn through a series of different solvents such as water, alcohol or acetone; in each of these the gas was soluble, imparting a strong smell to the solution. On passing the gas previously dried by quicklime and calcium chloride into concentrated sulphuric acid it was likewise absorbed, but the resulting solution was odourless, though on dilution with water the odour at once reappeared. This circumstance rendered possible the collection of a reserve of gas for subsequent examination; it was found, in fact, that the gas could be recovered unchanged from this solution after six months by dropping it carefully into ice-cold water.

On passing the dried gas into a dilute solution of bromine in carbon tetrachloride the colour of the bromine was rapidly discharged with the simultaneous formation of clusters of lemon-yellow glistening crystals. Pressed on a porous porcelain tile these crystals were found to be both volatile and deliquescent, and even when placed in a desiccator over phosphorus pentoxide they tended to disappear in the course of a short time. This substance was readily soluble in water, alcohol, ether and chloroform, but could be recrystallised from hot carbon tetrachloride, though not without some loss due to volatilisation, and melted with evolution of gas at 93–94°.

A small quantity of this substance, heated in a Carius tube for six hours to 300° with pure concentrated nitric acid, free from sulphuric acid, yielded a considerable quantity of sulphuric acid but not a trace of phosphoric acid, thus showing that it contained sulphur. A search in the literature for a bromine derivative of an alkyl sulphide having the melting-point above quoted proved unavailing, and being unfamiliar with the smell of alkyl sulphides I consulted Prof. Challenger who suggested that the smell of an aqueous solution of the gas evolved from the weed resembled that of methyl sulphide. I therefore procured from Messrs British Drug Houses an authentic sample of methyl sulphide and dissolved a little of it in water; on drawing air through the solution, drying the issuing gas with calcium chloride and passing it into a dilute solution of bromine in carbon tetrachloride as before, the same lemon-yellow crystals appeared; these crystals likewise melted at 93–94°, and when mixed with some of the crystals obtained from the natural source still melted at the same temperature, thus establishing the identity of the material prepared from the two sources. This circumstance could leave no doubt that the gas given off from *Polysiphonia* was in fact methyl sulphide.

On referring to the literature it was found that Cahours [1875] had obtained by the action of bromine on methyl sulphide in the presence of water a solution which on evaporation over sulphuric acid deposited amber-yellow transparent glistening octahedral crystals; he stated that the crystals were deliquescent but did not mention that they were volatile or that they had a melting-point. On repeating this preparation according to his description it was found that his crystals were in fact identical with these produced in carbon tetrachloride, in the absence of water, and had the same melting-point, 93–94°, which was not depressed by admixture with some of the crystals prepared from the *Polysiphonia* gas. It seems desirable therefore to put these facts on record and so amplify the data furnished by Cahours concerning this compound which he was the first to prepare.

With a view still further to confirm the identity of the substance evolved from *Polysiphonia fastigiata* the dried gas was passed into a saturated alcoholic solution of mercuric chloride and into an aqueous solution of potassium chloroplatinite. The precipitates obtained in each case were proved by their appearance and by their melting-points and mixed melting-points to be identical with the corresponding compounds prepared under identical conditions from the authentic sample of methyl sulphide.

DISCUSSION.

So far as I have been able to ascertain this is the first recorded case of the evolution of methyl sulphide in appreciable quantity from a plant. Traces of this substance have been found in the first runnings of the distillation of certain essential oils, such as American peppermint oil and the oils distilled from species of *Pelargonium* of African origin and from *Brassica juncea* seeds. In all these examples however the methyl sulphide occurs only in very small quantity and is not the sole or even the major constituent of the essential oil, and certainly cannot be detected in the smell of the original plant, as in the case of *Polysiphonia*.

It is worthy of note that the only other natural source of methyl sulphide recorded is a petroleum oil from Ohio described by Mabery and Smith [1891]. While the occurrence of the same substance in two such widely different materials as seaweed and mineral oil may be no more than a coincidence, it may at least be said that the finding of detectable quantities of methyl sulphide in a seaweed is not inconsistent with the theory of algal origin which has been suggested for some oil fields.

SUMMARY.

1. Methyl sulphide has been identified as the odoriferous principle evolved from *Polysiphonia fastigiata*.

2. The melting-point of the bromine derivative of methyl sulphide, originally prepared by Cahours, is recorded for the first time.

3. Attention is drawn to the other recorded natural occurrences of methyl sulphide.

I should like to express my acknowledgment for help both in collecting material and in some of the preliminary experimental work which was given by Mr W. K. H. Karstens during his tenure here of a grant from the Vollenhoven foundation of Leyden University.

REFERENCES.

Cahours (1875). *Compt. Rend. Acad. Sci.* **60**, 650.
Haas (1921). *Biochem. J.* **15**, 469.
—— and Russell-Wells (1923). *Biochem. J.* **17**, 696.
Mabery and Smith (1891). *Amer. Chem. J.* **13**, 237.

Note added May 23rd, 1935. Since writing the above I have been able to examine two other samples of *Polysiphonia nigrescens* and have found that they likewise gave off methyl sulphide.

CLVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

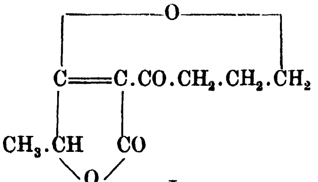
XLV. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH. IV. *l*-γ-METHYLTETRONIC ACID, WITH OBSERVATIONS ON THE FORMATION AND STRUCTURE OF RAMIGENIC AND VERTICILLIC ACIDS.

By PERCIVAL WALTER CLUTTERBUCK, HAROLD RAISTRICK AND FRITZ REUTER.

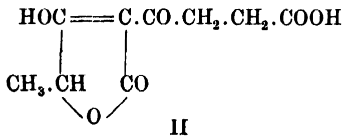
From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received April 13th, 1935.*)

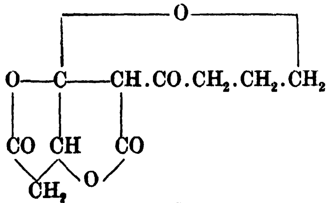
It was recently shown [Clutterbuck *et al.*, 1934] that *Penicillium Charlesii* G. Smith, when grown on nutrient solutions containing glucose, produces two new polysaccharides, mannocarolose [Haworth *et al.*, 1935] and galactocarolose, together with a number of new organic acids. The polysaccharides were isolated by precipitation from the evaporated metabolism solution with acetone, and the acids by solvent extraction of the filtrate from the polysaccharides, after removal of the acetone. Of these acids, carolic acid $C_9H_{10}O_4$ (I), carolinic acid $C_9H_{10}O_6$ (II), carlic acid $C_{10}H_{10}O_6$ (III), and carlosic acid $C_{10}H_{12}O_6$ (IV) have been shown [Clutterbuck *et al.*, 1935, 1, 2] to have the following molecular constitutions:



I



II



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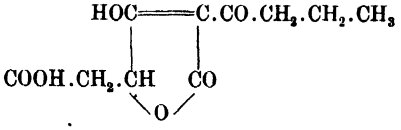
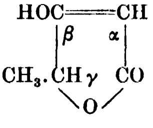


IV

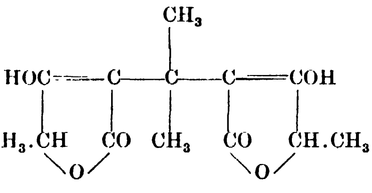
In addition to these four acids, two further acids, ramigenic acid $C_{16}H_{20}O_6$ and verticillic acid $C_{26}H_{32}O_{12}$ ($C_{26}H_{30}O_{11}.H_2O$), were also isolated in small amounts from the ether and chloroform extracts, using both Czapek-Dox and Raulin-Thom media.

During our investigation of the constitutions of carolic and carolinic acids, it was found that both acids on bromination gave *d*-α-bromo-γ-methyltetronic acid $C_5H_5O_3Br$, which on catalytic reduction gave *l*-γ-methyltetronic acid

$C_5H_6O_3$ (V). We have found that one of the most characteristic properties of *l*-γ-methyltetronic acid is the ease of its condensation with acetone. Thus on keeping a mixture of *l*-γ-methyltetronic acid and acetone in dilute aqueous solution at the ordinary temperature for 2–3 hours, especially in presence of HCl, or on boiling the mixture for a few minutes in absolute alcoholic solution, a practically quantitative yield of *iso*propylidene-bis-γ-methyltetronic acid (VI) is obtained.

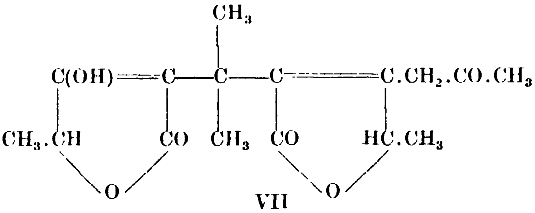


V



VI

If either of the above experiments is allowed to proceed for a longer time, a second molecule of acetone undergoes condensation and a beautifully crystalline acid, acetonyl*iso*propylidene-bis-γ-methyltetronic acid (VII) is formed. This acid has now been shown to be identical with ramigenic acid.



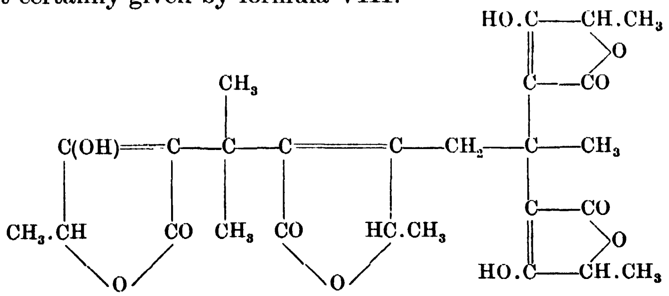
VII

Both of the above condensation reactions are strictly analogous to those obtained by Wolff and Schimpff [1901] with tetronic acid and acetone.

Further, we have found that verticillic acid (1 mol.) on standing overnight in dilute HCl, or more rapidly in acetic acid solution at room temperature is hydrolysed and gives 1 mol. of ramigenic acid and 2 mols. of *l*-γ-methyltetronic acid:

$$C_{26}H_{30}O_{11}.H_2O \longrightarrow C_{16}H_{20}O_6 + 2C_5H_6O_3$$

From its general properties, the structure of anhydrous verticillic acid is therefore almost certainly given by formula VIII.



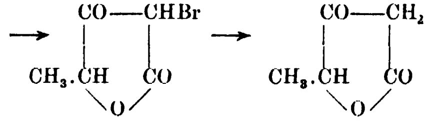
VIII

In view of the fact that in the early work the polysaccharide fraction had been separated by precipitation with acetone (alcohol had been avoided because slight esterification of the metabolic acids occurred) it appeared a reasonable supposition that verticillic and ramigenic acids were artefacts arising from *l*-γ-methyltetronic acid by reaction with acetone during the separation of the polysaccharide fraction. That this view is correct has now been proved by repeating the metabolism experiments exactly as in our first experiments but replacing acetone with alcohol as a means of precipitation of the polysaccharide fraction. The combined ether extracts of the alcohol- and polysaccharide-free metabolism fluid then gave an oil from which *l*-γ-methyltetronic acid was isolated in a yield of the same order as would be expected from the previously obtained yields of ramigenic and verticillic acids, assuming that these acids were formed by reaction of 2 and 4 mols. respectively of *l*-γ-methyltetronic acid with acetone. Further, ramigenic and verticillic acids were entirely absent, while, on the other hand, carolic, carolinic, carlic and carlosic acids were isolated from the ether and chloroform extracts of the metabolism solution by methods similar to those previously employed, and in comparable yields.

Carolic, carolinic, carlic, carlosic and *l*-γ-methyltetronic acids should therefore be regarded as true metabolism products, whilst the ramigenic and verticillic acids of our earlier experiments must now be regarded as artefacts arising from *l*-γ-methyltetronic acid by condensation with the acetone used for precipitation of the polysaccharide fraction.

During this work a small amount of synthetic γ-methyltetronic acid was required for comparison with our natural product. In our earlier paper, the method of Benary [1911] was used for its synthesis. This method, although repeated several times under varying conditions, always gave only a very small yield of γ-methyltetronic acid and a new synthesis was therefore elaborated. Ethyl propionylacetate was first synthesised by a Grignard condensation of ethyl iodide and ethyl cyanoacetate with subsequent hydrolysis and was then brominated under conditions which gave the αγ-dibromo-derivative of this ester. This compound on heating *in vacuo* readily lost ethyl bromide with ring closure and formation of α-bromo-γ-methyltetronic acid, $C_5H_5O_3Br$, which on catalytic reduction was converted into *dl*-γ-methyltetronic acid, $C_5H_6O_3$:

$$CH_3.CH_2.CO.CH_2.COOEt \longrightarrow CH_3.CHBr.CO.CHBr.COOEt$$



The acid obtained was in every way identical with the acid synthesised by Benary's method. Like the latter it did, however, give a slight depression of M.P. with the *l*-variety of this acid, M.P. 115° obtained by degradation of carolic and carolinic acids.

EXPERIMENTAL.

Earlier experiments.

In our previously described experiments, the polysaccharide fraction was separated from the evaporated metabolism solution by precipitation with acetone, the mother-liquors from which were concentrated to 600 ml., acidified with concentrated HCl and extracted several times first with ether and subsequently with $CHCl_3$.

The material extracted with ether, on grinding with a small volume of $CHCl_3$, filtering and washing with $CHCl_3$, gave a crystalline powder consisting in the

Czapek-Dox experiments of carlosic and carolinic acids, and in the Raulin-Thom experiments of carolinic and verticillic acids. The $CHCl_3$ filtrates from these acids, after removal of solvent, gave syrups which in both cases were ground with water. The fraction soluble in water was extracted with $CHCl_3$ and gave a yellow oil which slowly crystallised and consisted chiefly of carolic acid, whilst the material insoluble in water gave, on standing, crystals of ramigenic acid, and the residual syrups on neutralising with NaOH and adding $HgCl_2$ gave a precipitate of the mercuric salt of verticillic acid.

The material extracted with $CHCl_3$ was in the first extract almost pure carolic acid and in the remaining extracts carlic acid.

Present experiments.

In the present experiments the initial procedure was the same as described above except that absolute alcohol replaced acetone as the polysaccharide precipitant. After filtering off the polysaccharide fraction and concentrating *in vacuo* at 40–45° to remove alcohol, the mother-liquors were extracted as before with ether and chloroform.

The yellow syrups obtained by ether extraction of the concentrated metabolism solutions using Raulin-Thom and Czapek-Dox medium respectively should (according to our earlier work) contain carolinic acid, some carolic acid ramigenic and verticillic acids, and from Czapek-Dox medium carlosic acid in addition. This fraction also should contain most of the *l*-γ-methyltetronic acid if this acid were present. Of all these acids only *l*-γ-methyltetronic, ramigenic and verticillic acids give insoluble mercuric salts. In the present series of experiments therefore the ether-extracted material was divided in the case of each medium into two equal portions. One portion was neutralised with NaOH and treated with a large excess of saturated $HgCl_2$. The precipitate consisted almost entirely of the mercuric salt of *l*-γ-methyltetronic acid, ramigenic and verticillic acids being entirely absent. The second portion of the ether-extracted material was examined exactly as in our earlier experiments for the presence of carolinic, carlosic and carolic acids.

The material extracted with $CHCl_3$ was separated, as in our earlier experiments, into carolic and carlic acids.

Isolation of the products of metabolism of Penicillium Charlesii *G. Smith using ethyl alcohol as carbohydrate precipitant.*

One hundred 1-litre conical flasks containing 350 ml. Czapek-Dox solution and a similar number of flasks of Raulin-Thom solution were sterilised and sown as in our earlier paper [Clutterbuck *et al.*, 1934] with a spore suspension of *P. Charlesii* and incubated at 24° until the glucose had just disappeared. This occupied about 7 weeks with Czapek-Dox and about 4 weeks with Raulin-Thom medium. The metabolism solution was filtered from the mycelium and the latter well pressed and washed with water. The filtrate and washings were evaporated *in vacuo* at 45–50° to about 800 ml. and kept for 1 day at 0°. A small amount of amorphous material was filtered off and to the filtrate were added 4 volumes of absolute alcohol, the mixture being well stirred and allowed to stand until the precipitated polysaccharide mixture had completely settled. The clear liquid was then decanted and evaporated *in vacuo* to about 600 ml. to remove the alcohol. To the concentrate 125 ml. concentrated HCl were added, the fluid was extracted 10 times with an equal volume of ether, the combined ethereal extracts evaporated and the extracted material dried *in vacuo*. In this way were obtained, using Czapek-Dox medium, 153·2 g. and with Raulin-Thom medium 87·2 g. of a yellow oil.

The ether-extracted solution was then re-extracted with chloroform for the separation of carolic and carlic acids. The number of extracts was increased to 25 and this, together with the fact that a larger amount of HCl had been employed for acidification, led to the isolation of almost twice as much carlic acid as had been previously recorded. It was noticed in our earlier experiments that even after protracted extraction with ether and chloroform the mother-liquors still retained a small negative rotation, and this is now shown undoubtedly to be due to the incomplete extraction of carlic acid. The weights, after drying, of the first $CHCl_3$ extract were, on Czapek-Dox medium, 29·8 g. and on Raulin-Thom medium 24·9 g., whilst the remaining 24 extracts of the former medium weighed 17·5 g. and of the latter 20·0 g.

The ether extract was next divided into two equal portions, half being examined by the mercury salt method (*a*) for the presence of *l*-γ-methyltetronic, verticillic and ramigenic acids, and the remaining half being worked up (*b*) for carolic, carolinic and carlosic acids as in our earlier experiments.

(*a*) *Isolation of* l-γ-*methyltetronic acid as a product of metabolism of* P. Charlesii. The half-portions of ether-extracted material from experiments on each medium were first neutralised with NaOH using litmus paper as an external indicator, treated with a large excess of aqueous $HgCl_2$ and kept overnight. The resultant precipitate was separated by centrifuging, washed in the centrifuge pots, suspended in water and saturated with H_2S. The HgS was filtered off, H_2S removed from the filtrate in a stream of air and the free acids extracted with ether. On removing the ether a product was obtained which immediately crystallised but contained a little yellow oil. The total weight of this crude product with Czapek-Dox medium was 6·9 g. and with Raulin-Thom medium 12·6 g. The products were rubbed up with a little ether, transferred to a filter and washed with ether. The yellow oil was thus completely removed and dry crystalline powders, weights 4·5 and 9·2 g. respectively, remained. The material in both cases melted at 114° and consisted of almost pure *l*-γ-methyltetronic acid. No ramigenic or verticillic acid was present. After sublimation the *l*-γ-methyltetronic acid melted at 115°. (Found (Schoeller): C, 52·63, 52·61; H, 5·27, 5·28 %. $C_5H_6O_3$ requires C, 52·61; H, 5·30 %.)

0·066 g. required 5·80 ml. $N/10$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 114 ($C_5H_6O_3$ titrating as a monobasic acid requires 114). The free acid in water ($c=0·526$) had $[\alpha]_{5461} - 21°$. The rotation was therefore identical with that of the acid obtained from carolic and carolinic acids by bromination followed by catalytic reduction of the resultant *d*-α-bromo-γ-methyltetronic acid.

The acid obtained both from Czapek-Dox and Raulin-Thom media did not depress the M.P. of a sample of *l*-γ-methyltetronic acid obtained from carolic acid, and gave red $FeCl_3$ and violet $NaNO_2$ colours and a mono-2:4-dinitrophenylhydrazone, M.P. 235°, not depressed by mixing with the corresponding dinitrophenylhydrazone of synthetic *dl*-γ-methyltetronic acid. A neutral solution of the acid gave with $FeCl_3$ a very intense brownish red colour similar to that noted in our earlier paper with a neutral solution of verticillic acid.

It is certain therefore that when alcohol is used to precipitate the polysaccharide fraction, *l*-γ-methyltetronic acid can be isolated as a true metabolism product, whilst the ramigenic and verticillic acids of our earlier experiments are entirely absent and have probably arisen as artefacts by condensation of *l*-γ-methyltetronic acid with the acetone then used as polysaccharide precipitant.

(*b*) *Isolation of carolic, carolinic and carlosic acids from the ether extract.* This material, which after standing was for the most part crystalline, was ground in a glass mortar with a small amount (20 ml.) of chloroform, filtered and washed with chloroform. In this way the ether extract from each medium was separated into a quantity of light brown crystalline material and a chloroform mother-liquor which, after removal of solvent, was dried down to a syrup.

Using Czapek-Dox medium, the crystalline fraction consisted of a mixture of carlosic and carolinic acids. These acids were separated by boiling with a large volume of benzene and filtering, when carlosic acid immediately separated on cooling, whilst carolinic acid remained for the most part in solution and was again obtained by concentrating the benzene solution, adding a little light petroleum and setting aside to crystallise. Carlosic acid after several recrystallisations from boiling benzene had M.P. 181°, not depressed by admixture with an authentic specimen. The carolinic acid fraction was recrystallised once from a small volume of water and then several times from acetone-light petroleum and melted at 123°, not depressed by admixture with an authentic specimen.

Using Raulin-Thom medium, the crystalline fraction consisted almost entirely of carolinic acid, which after one recrystallisation from a small volume of water and then from acetone-light petroleum had M.P. 123°, not depressed by admixture with an authentic specimen. It will be noted that in our earlier experiments verticillic acid accompanied carolinic acid in this fraction but was now absent.

The syrups remaining after drying down the chloroform washings from the crystalline fractions referred to above contained in addition to a reddish brown oil both carolic and *l*-γ-methyltetronic acids. Thus on grinding half of this syrup with water, extracting the aqueous phase once with chloroform and removing the chloroform, a pale yellow oil remained which on standing slowly crystallised. This material was rubbed up with a small volume of alcohol, filtered, washed with a small volume of alcohol and finally recrystallised from this solvent. It had M.P. 132°, not depressed on admixture with an authentic specimen of carolic acid. The presence of *l*-γ-methyltetronic acid in the remaining portion of the syrup was shown by the mercury salt method used above in its isolation.

(*c*) *Isolation of carolic and carlic acids from the chloroform extract.* The isolation of carolic and carlic acids from the material extracted by $CHCl_3$ from the ether-extracted, carbohydrate-free metabolism solution was carried out exactly as in our earlier paper. Thus the semi-crystalline extracts were ground with a small amount (20 ml.) of absolute ethyl alcohol, filtered and washed with a little alcohol. In this way the first $CHCl_3$ extract using both Czapek-Dox and Raulin-Thom media gave almost pure carolic acid which after recrystallisation from alcohol had M.P. 132°, not depressed by admixture with an authentic specimen. The material from the remaining 24 $CHCl_3$ extracts on the other hand gave almost pure carlic acid, which after recrystallisation from a small volume of ethyl alcohol had M.P. 176°, not depressed by admixture with an authentic specimen.

The total yields in g. of the almost pure crystalline acids obtained from 100 flasks are given in the following table. The yields of *l*-γ-methyltetronic,

Medium	Carolic acid $C_9H_{10}O_4$	Carolinic acid $C_9H_{10}O_6$	Carlic acid $C_{10}H_{10}O_6$	Carlosic acid $C_{10}H_{12}O_6$	*l*-γ-Methyltetronic acid $C_5H_6O_3$
Czapek-Dox	27·2	6·5	19·3	6·3	4·5
Raulin-Thom	17·2	14·7	5·1	None	9·2

carlosic, carolinic and carolic acids actually isolated from half of the ether extracts have been doubled so as to refer to the total ether extract.

These figures are of the same order as in our earlier experiments except that the yield of carlic acid on Czapek-Dox medium is higher, owing to more thorough extraction, whilst the yields of verticillic and ramigenic acids obtained in the earlier experiments are replaced by a yield of *l*-γ-methyltetronic acid roughly corresponding to the combined weights of these two acids.

Hydrolysis of verticillic acid to give ramigenic and l-*γ-methyltetronic acids.*

This hydrolysis occurs only slowly in dilute HCl solution at room temperature because of the extremely small solubility of verticillic acid. The reaction was readily effected in the following way. Verticillic acid hydrate (VIII) (2 g.) was dissolved in 64 ml. 8 % aqueous sodium acetate solution, diluted to 100 ml. and 2*N* HCl added to faint turbidity (14 ml.). The solution was kept overnight. Large colourless prisms (1·12 g., M.P. 166–7°) separated, were filtered off and shown to be the hydrate of ramigenic acid (VII). Thus after recrystallisation from acetone-light petroleum the acid had M.P. 171°, not depressed on admixture with an authentic specimen of ramigenic acid, but depressed 10–12° on admixture with verticillic acid (M.P. 171°). It gave the characteristic rotation values of ramigenic acid, the anhydrous free acid in alcohol having $[\alpha]_{5461}+28°$ and the sodium salt $[\alpha]_{5461}+85°$. (Found (Schoeller) on anhydrous material: C, 62·34, 62·27; H, 6·47, 6·59 %. $C_{16}H_{20}O_6$ requires C, 62·30; H, 6·54 %.) The acid also gave on methylation the characteristic monomethyl derivative of ramigenic acid. Thus on suspending the finely powdered acid in ether and adding an ethereal solution of diazomethane there was an immediate and vigorous reaction and a methyl derivative separated, M.P. 192°. On dissolving in a larger volume of boiling ether, filtering and adding an equal volume of boiling light petroleum, the methyl derivative separated in long colourless needles, M.P. 199°. (Found: C, 63·41, 63·47; H, 6·91, 6·93; OMe, 9·60, 9·78 %; Mol. wt. (cryoscopic in camphor) 332, 340. $C_{16}H_{19}O_5OCH_3$ requires: C, 63·31; H, 6·88; OMe, 9·62 %. Mol. wt. 322.)

The mother-liquors from which ramigenic acid had been separated were continuously extracted with ether, and the extracted material, which contained some acetic acid, was rapidly dried over sticks of KOH at very low pressure. A solid remained (0·90 g.) which contained a trace of yellow oil but consisted essentially of colourless crystalline material. This sublimed with great ease and after two sublimations had M.P. 115°, not depressed by admixture with a sample of *l*-γ-methyltetronic acid from carolic acid. (Found: C, 52·82, 52·80; H, 5·37, 5·37 %. $C_5H_6O_3$ requires C, 52·61; H, 5·30 %.) 0·052 g. required 4·60 ml. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 113. ($C_5H_6O_3$ titrating as a monobasic acid requires 114.) This acid gave red $FeCl_3$ and violet $NaNO_2$ colours and a mono-dinitrophenylhydrazone, M.P. 235°, not depressed by mixing with the corresponding dinitrophenylhydrazone of synthetic *dl*-γ-methyltetronic acid. (Found: C, 45·03, 45·18; H, 3·47, 3·57; N, 19·04, 19·01 %. $C_{11}H_{10}O_6N_4$ requires C, 44·89; H, 3·43; N, 19·04 %.)

The theoretical yield from 2 g. of verticillic acid hydrate is 1·22 g. of ramigenic acid $C_{16}H_{20}O_6.H_2O$ (assumed 1 mol.), and of *l*-γ-methyltetronic acid (assumed 2 mols.) is 0·85 g. It appears therefore that verticillic acid (1 mol) on mild hydrolysis gives rise to 1 mol. of ramigenic acid and 2 mols. of *l*-γ-methyltetronic acid. This fact fixes the empirical formula of verticillic acid hydrate as $C_{26}H_{32}O_{12}$, and confirms the formula previously assigned to this substance.

Synthesis of ramigenic acid and iso*propylidene-bis-γ-methyltetronic acid.*

For this purpose the natural *l*-γ-methyltetronic acid obtained as a metabolic product of *P. Charlesii* and also a sample of the same acid prepared from carolic acid by bromination and reduction of the so-formed α-bromo-γ-methyltetronic acid were used.

l-γ-Methyltetronic acid on standing in dilute aqueous solution with acetone for 2–3 hours readily deposits crystals of *iso*propylidene-bis-γ-methyltetronic acid (VI). After filtering off this compound, the mother-liquors on keeping for 48 hours deposit crystals of ramigenic acid (VII). Both of these reactions proceed somewhat more quickly in presence of HCl. The reactions can be best carried out by boiling a solution of *l*-γ-methyltetronic acid (0·25 g.) in absolute alcohol (5 ml.) containing acetone (5 ml.) on the water-bath under reflux. If the reaction is stopped after 30 minutes, the acetone distilled off and water added, *iso*propylidene-bis-γ-methyltetronic acid crystallises out. If boiling is continued for 4–5 hours, the product is pure ramigenic acid. An exact analogy for both of these reactions is given by Wolff and Schimpff [1901] in their reactions of tetronic acid and acetone which give *iso*propylidene-bis-tetronic acid and acetonyl-*iso*propylidene-bis-tetronic acid respectively.

Iso*propylidene-bis-γ-methyltetronic acid* (VI) crystallises from water with $2H_2O$, which is readily lost on heating at 63° *in vacuo*. The anhydrous acid melts at 172°. (Found (Schoeller): C, 58·09, 58·18; H, 6·09, 6·00 %. $C_{13}H_{16}O_6$ requires C, 58·19: H, 6·02 %.) The acid in alcohol ($c=0{\cdot}513$) had $[\alpha]_{5461} - 39°$.

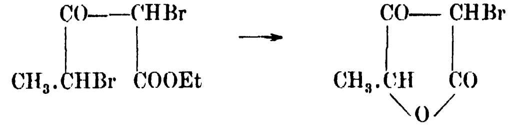
0·0298 g. required 2·19 ml. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 136 ($C_{13}H_{16}O_6$, titrating as a dibasic acid requires 134). The acid gives a red $FeCl_3$ colour in aqueous alcohol but does not give an immediate colour with $NaNO_2$.

*Acetonyl*iso*propylidene-bis-γ-methyltetronic acid* (ramigenic acid, VII) synthesised as described above showed exactly the same properties as those of ramigenic acid as described in our earlier paper. Both acids crystallised in the same characteristically well-formed prisms, M.P. 171°, the mixed M.P. showing no depression. Both acids can be crystallised both in the anhydrous form $C_{16}H_{20}O_6$ and as the monohydrate. Both acids show the same abnormality in rotation. Thus in each case the free acid in absolute alcohol ($c=0{\cdot}22$) had $[\alpha]_{5461} + 28°$ whereas the sodium salt (calculated as monosodium salt) in water ($c=0{\cdot}27$) had $[\alpha]_{5461} + 85°$. Both acids when suspended in ether in finely powdered condition reacted immediately with an ethereal solution of diazomethane and gave the same monomethyl derivative, which after recrystallising from ether-light petroleum melted at 199°, the mixed M.P. showing no depression. The synthetic acid showed the same solubilities and behaviour on titration and gave the same reactions with salt solutions as were described in our earlier paper for ramigenic acid. It is certain therefore that the synthetic acetonyl*iso*propylidene-bis-γ-methyltetronic acid (VII) is identical with the ramigenic acid isolated in earlier experiments from the metabolism fluid and the ease of its formation in dilute aqueous solutions containing *l*-γ-methyltetronic acid and acetone makes it certain that the ramigenic acid of our earlier experiments was an artefact arising from these substances during the separation of the polysaccharide fraction with acetone.

A new synthesis of dl-*γ-methyltetronic acid.*

In our earlier paper [Clutterbuck *et al.*, 1935, 1] this acid was synthesised by the method of Benary [1911] by condensation of bromopropionylbromide with ethyl sodiomalonate with the usual ring closure by loss of ethyl bromide on heating and subsequent hydrolytic decarboxylation. With this method however the yield we obtained was always very small and a better method was desirable.

In our new method, ethyl propionylacetate was first synthesised by the Grignard reaction of Blaise [1901] as modified by Willstätter and Clarke [1914]. This ester was then brominated under conditions, which by analogy with the work of Wolff [1896] should result in the formation of ethyl $\alpha\gamma$-dibromopropionylacetate. Ring closure was then effected by heating as usual giving α-bromo-γ-methyltetronic acid,



which on catalytic reduction with palladium-charcoal-hydrogen readily gave *dl*-γ-methyltetronic acid.

Ethyl propionylacetate (11 g.) was dissolved in ether (100 ml.) and 7·8 ml. bromine ($\equiv$4 atoms Br) were slowly added, the flask being cooled in running water. The reaction was immediate. The ether layer was washed with water until free from HBr, dried over $MgSO_4$ and the ether removed *in vacuo*. The resulting dibromoester was highly lachrymatory and had a very disagreeable odour. It was not therefore isolated in the pure state but was immediately heated at reduced pressure (about 30 mm.) for 3 hours in an oil-bath, temperature 130–140°. In this way ring closure with loss of ethyl bromide was effected. On cooling the product set to a semi-crystalline mass and was kept overnight in the cold room. On filtering and washing with benzene 3·35 g. of crystalline α-bromo-γ-methyltetronic acid remained. The filtrate, which on adding benzene deposited a small amount of tar, was filtered and evaporated and the residue heated for a further period as above. On standing in the cold room overnight, filtering and washing with benzene, a second crop of the bromide (0·62 g.) was obtained. The total yield of α-bromo-γ-methyltetronic acid was thus 3·97 g. or 26·8 % of theory. The product melted at 173° and gave red $FeCl_3$ and violet $NaNO_2$ colour reactions. It was reduced catalytically with palladium-charcoal-hydrogen in the same way as were the bromides from carolic and carolinic acids in our earlier work, and gave quantitatively *dl*-γ-methyltetronic acid $C_5H_6O_3$, which crystallised from benzene-light petroleum in curved feathery needles, M.P. 117°. It readily sublimed *in vacuo* and the sublimate melted at 117°. (Found (Weiler): C, 52·94; H, 5·69 %. $C_5H_6O_3$ requires C, 52·61; H, 5·30 %.) 0·0745 g. required 6·60 ml. $N/10$ NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 113 ($C_5H_6O_3$ titrating as a monobasic acid requires 114).

With Brady's reagent it readily gave a crystalline mono-dinitrophenylhydrazone, M.P. 235°, unchanged by recrystallisation from nitrobenzene-toluene and not depressed by admixture with the dinitrophenylhydrazone from the acid prepared by earlier methods. The acid on boiling with $2N$ H_2SO_4 was readily hydrolysed giving CO_2, and the hydrolysate slowly, after the manner of acetoin, gave the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°, not depressed by admixture with an authentic specimen.

The acid $C_5H_6O_3$ is therefore undoubtedly the *dl*-variety of γ-methyltetronic acid.

SUMMARY.

It is shown that *l*-γ-methyltetronic acid $C_5H_6O_3$ is formed as a metabolic product of *Penicillium Charlesii* G. Smith when this mould is grown on synthetic solutions containing glucose. A new synthesis of the *dl*-form of this acid is described.

Ramigenic acid, $C_{16}H_{20}O_6$, and verticillic acid, $C_{26}H_{32}O_{12}$, previously reported as metabolic products of *P. Charlesii*, are now shown to be artefacts arising by condensation of *l*-γ-methyltetronic acid with the acetone previously used for precipitation of the polysaccharide fraction from the evaporated metabolism solution. Ramigenic acid has been synthesised from *l*-γ-methyltetronic acid and acetone and shown to be acetonyl*iso*propylidene-bis-γ-methyltetronic acid. Verticillic acid has been shown to be the bis-γ-methyltetronic acid derivative of ramigenic acid.

REFERENCES.

Benary (1911). *Ber. deutsch. chem. Ges.* **44**, 1763.
Blaise (1901). *Compt. Rend. Acad. Sci.* **132**, 978.
Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.
—— Raistrick and Reuter (1935, 1). *Biochem. J.* **29**, 300.
—— —— —— (1935, 2). *Biochem. J.* **29**, 871.
Haworth, Raistrick and Stacey (1935). *Biochem. J.* **29**, 612.
Willstatter and Clarke (1914). *Ber. deutsch. chem. Ges.* **47**, 298.
Wolff (1896). *Liebig's Ann.* **291**, 231.
—— and Schimpff (1901). *Liebig's Ann.* **315**, 164.

CLIX. PHOSPHATE AND CREATINE METABOLISM IN FEVER.

BY WILFRID WALTER PAYNE.

From the Biochemical Department, The Hospital for Sick Children, Great Ormond Street, W.C. 1.

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WHILE making some observations in a case of juvenile G.P.I. undergoing malaria treatment it was noted that some urine specimens were practically devoid of phosphate. A search through recent text books and articles failing to give any reference to this phenomenon it was decided to study the problem further.

METHOD.

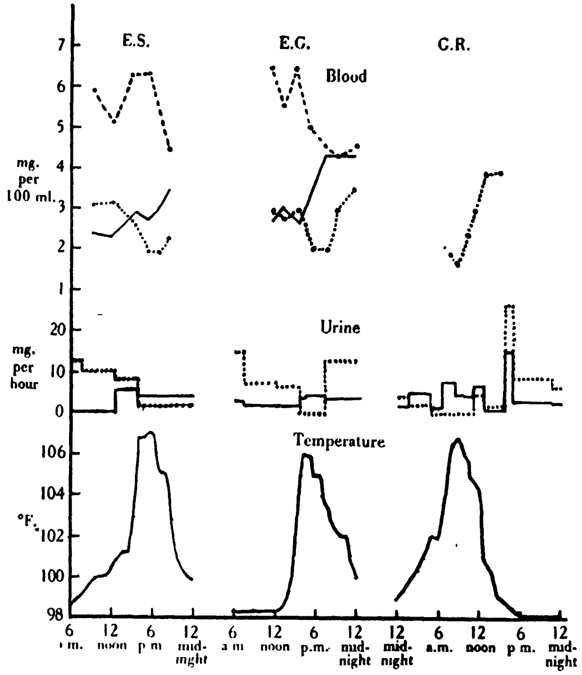
When the children had been infected with malaria by mosquitoes there was an incubation period of from 10–21 days. During this period observations on the blood and urine were made. The total urinary output was collected in three daily periods 7 a.m.–1 p.m., 1 p.m.–7 p.m., 7 p.m.–7 a.m. (the times arranged to suit the convenience of nursing), and the various estimations performed daily. This was continued until the pyrexia developed. From this point the whole of every specimen of urine passed was collected separately by the nurse and the time noted. Blood examinations were made at suitable times. This was continued until the attack was terminated by quinine administration. The collection of urine was continued until more or less normal figures were obtained. A further blood examination was then made. In no single case however was it possible for the complete programme to be carried out.

The analytical methods used were: urea—McLean; phosphate (blood and urine)—Briggs; creatine and creatinine (blood and urine)—Folin; blood sugar—micro-Folin-Wu. By simple adaptations of the blood methods it was found possible to do most of the investigations on about 1–1·5 ml. blood which was obtained from a single puncture of a finger. It was found that this disturbed the children far less than puncture of a vein. Even so it was not considered advisable to take as frequent specimens as could have been desired.

RESULTS.

Urine. Two sets of changes were observed—a gradual alteration of the total daily output of phosphate and creatine as the malaria infection increased in intensity and a rapid alteration during the pyrexial bouts. These changes are shown in Figs. 1 and 2. In Fig. 2 the relation between the hourly excretion of phosphate and creatine is obscured on a few occasions by a profuse polyuria which produced high excretion values for all the urinary constituents.

Phosphate. The daily phosphate excretion diminishes with the duration of the fever until the termination either spontaneously or after quinine. Also during each bout of temperature the phosphate excreted diminishes, the maximum fall occurring at the time of the peak temperature. When the fever is well established the phosphate in the urine completely disappears. (No colour was



Blood: ----- creatine; —— creatinine; ····· phosphate.
Urine: ..... phosphate; —— creatine.

Fig. 1. The changes in blood and urine during one pyrexial period in three separate subjects. Blood: mg. per 100 ml. Urine: mg. per hour.

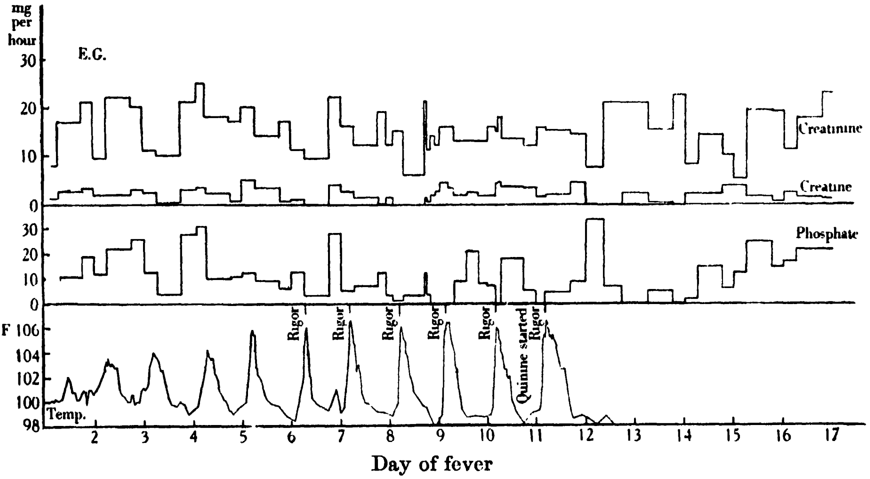


Fig. 2. The hourly excretion rate of phosphate, creatine and creatinine in a subject over the duration of an attack of malaria.

formed with Briggs's reagents even with much extra urine.) Immediately after the peak the phosphate rises often to quite high values.

Creatine. The general level of creatine excretion falls somewhat with the full onset of the fever and then returns to normal. After quinine the creatine again

Table I, A, B and C. *To show the variation in the blood and urine phosphate and creatine with change of temperature.*

Date	Time	Temp. °C.	Phosphate mg.P/ 100 ml.	Creatine mg./ 100ml.	Creatinine mg./ 100ml.	Sugar mg./ 100ml.	Urinary P	Urinary Creatine
				A.				
E. S.								
20. x. 32	10.50 a.m.	99·0	3·45	6·1	2·6	99	Normal	Normal
	3.40 p.m.	99·4	3·45	7·9	2·8	132	Normal	—
25. x. 32	11.50 a.m.	103·6	3·2	7·2	2·7	93	Low	Normal
Peak	3.30 p.m.							
	3.50 p.m.	104·4	3·0	4·5	3·8	91	Low	Normal
27. x. 32	11.0 a.m.	105·0	2·1	9·2	4·1	95	Normal	Normal
	12.15 p.m.	105·6	2·5	7·6	2·6	105	Low	High
	2.0 p.m.	105·8	1·8	6·7	3·3	121	Low	High
Peak	12.30–2.30							
	4.35 p.m.	104·6	2·6	6·7	2·9	99	Normal	High
2. xi. 32	9.30 a.m.	98·6	3·1	5·8	2·4	100	Normal	None
	11.30 a.m.	100·2	3·15	5·1	2·3	100	Normal	None
	3.40 p.m.	105·4	2·6	6·3	2·9	107	Low	Normal
	5.0 p.m.	106·6	1·95	6·3	2·7	105	Low	Normal
Peak	5.30 p.m.							
	6.30 p.m.	106·0	1·9	5·5	3·0	110	Low	Normal
	8.0 p.m.	104·8	2·25	4·5	3·5	100	Low	Normal
3. xi. 32	11.0 a.m.	98·8	4·05	5·6	2·6	103	Normal	Normal
Quinine given 4.15 p.m. 3. xi. 32								
4. xi. 32	3.40 p.m.	99·2	2·4	5·2	2·1	97	Normal	Normal
5. xi. 32	11.30 a.m.	101·0	2·3	5·6	2·0	114	None	Very low
7. xi. 32	11.20 a.m.	98·4	2·7	5·7	2·0	83	Normal Prev. spec. very low	Normal Prev. spec. none
8. xi. 32	11.45 a.m.	98·4	3·4	6·0	1·9	135	Low Prev. spec. very low	Low Prev. spec. none
				B.				
E. G.								
20. iv. 32	11.30 a.m.	98·4	2·9	6·2	2·8	93	Normal	Low
	1.0 p.m.	98·4	2·9	5·3	3·0	75	Lower	Low
	3.0 p.m.	101·0	3·1	6·5	2·8	95	Lower	Low
	5.0 p.m.	106·0	2·0	5·1	3·9	93	None	Low
Peak	5.0 p.m.							
	7.0 p.m.	103·9	2·0	4·8	4·2	88	None	Normal
	9.0 p.m.	102·0	3·0	4·1	4·1	94	No spec. till 6.0 a.m.	
	11.0 p.m.	100·8	3·5	4·3	4·1	85		
21. iv. 32	10.30 a.m.	98·4	2·8	5·9	3·1	80	High	Normal
26. iv. 32	?	98·4	3·7	9·0	3·0	98	Normal	Normal
				C.				
G. R.								
14. i. 32 Peak	8.30 a.m.							
	9.40 a.m.	106·5	1·5	—	—	166	None	Normal
	1.0 p.m.	105·2	—	—	—	139	None	Normal
	2.45 p.m.	102·0	4·4	—	—	135	Low	Normal
15. i. 32	3.50 p.m.	98·4	2·8	—	—	124	Normal	Normal
16. i. 32	7.15 a.m.	106·5	1·9	—	—	—	Trace	Normal
	8.15 a.m.	106·8	1·7	—	—	127	Trace	Increasing
Peak	8.15 a.m.							
	9.15 a.m.	106·0	1·9	—	—	93	None	Raised
	10.15 a.m.	105·0	1·5	—	—	93	None	Raised
	11.15 a.m.	104·5	3·0	—	—	110	None	Raised
	12.20 p.m.	102·5	3·8	—	—	104	Low	Raised
	1.15 p.m.	100·8	3·9	—	—	115	Increasing	Low
	2.35 p.m.	98·8	3·9	—	—	99	Increasing	Low

falls. The creatine varies with the temperature. At the time of the rise of temperature there is a rise in the creatine output. A fall starts just after the peak and the creatine output reaches its lowest level at the time when the temperature has just returned to normal. When this coincides with the general low level of excretion no creatine is found in the urine. A similar absence of creatine from the urine occurs after quinine. During the febrile period the minimum values of phosphate and creatine in the urine never coincide, but after quinine they may occur together.

Creatinine. The changes in creatinine excretion are less clear cut. The excretion often runs more or less parallel with that of creatine but at a higher level. There is a tendency for a rise above normal to occur with the rise of temperature but the fall which occurs in the intervals is only slightly below the normal level.

Blood. The changes in the blood are shown in Figs. 1 and 2 and in tabular form in Table I, A, B and C.

Inorganic phosphorus. The blood phosphorus falls to quite low values by the time the peak temperature is reached. The fall starts at the same time as the rise of temperature. There is a corresponding rise in the phosphorus when the peak is passed. The exact time of this rise varies in different cases from the peak to 2 hours after the peak. After quinine low values were obtained.

Creatinine and creatine. The creatine content of the blood rises as the temperature rises. After the peak there is an abrupt fall. The post-critical rise of the blood phosphate does not occur until this fall is complete. Figures are not available for the values in the interval, several days elapsing in every case before another set of observations was made. After quinine all values obtained were somewhat lower than the pre-febrile ones.

Blood sugar. Although the blood sugar was estimated frequently no correlation could be observed between the fever and the blood sugar. The added variable of food which was taken irregularly in relation to the pyrexia and absorbed probably equally irregularly would be sufficient to obscure any underlying relationship unless it were very marked.

These changes are not found solely in malaria since other conditions in which similar bouts of pyrexia alternate with periods of normal temperature also show similar changes. They were shown in a boy suffering from streptococcal septicaemia and to less extent in a child in whom pyrexia was being produced by repeated T.A.B. injections intravenously.

Discussion.

These very striking changes in the phosphate of the urine have been reported as far back as 1860 in malaria by Haxthausen [1860] and many others. A survey of the literature up till 1900 has been made by Garratt [1904]. The findings of all the observers when dealing with malaria were uniform and identical with those reported here. In dealing with non-malarial cases no uniformity of results was obtained, there being no periodic swing to reveal such changes as occur in malaria. The daily output was either slightly increased or diminished at the start and suffered far less variation than did the urea.

No conclusions as to the cause of the changes were advanced by Garratt, but in his discussion he suggests that the phosphorus may have been stored in the muscles and glands of the body. Indeed, no adequate answer could have been given until the recent work on muscle metabolism culminated in the simultaneous discovery of phosphocreatine by Eggleton and Eggleton [1927], and by Fiske and Subbarow [1927].

The precritical period is marked by a rapid rise of temperature and by muscular tremors or rigors which should produce effects similar to muscular work. The effect of work is somewhat different according to its duration and to the degree of fatigue. Short sharp exercise (10 secs.) produces only a fall of blood phosphorus after a few minutes' interval [Gemmill and Ribeiro, 1933], but after exercise of a longer duration (20 mins.) causing exhaustion there is a rise of blood phosphorus followed by a fall. Corresponding but less marked changes occur in the urine, the rise being greater than the fall [Havard and Reay, 1926; Planet and Cardoso, 1933]. There is also a rise in the blood creatine [Kael, 1932; Planet and Cardoso, 1933]. Tetanic stimulation of excised muscle increases the hexosephosphate, but this increase rapidly disappears on cessation of the stimulus [Cori and Cori, 1931; 1933].

Apart from work, adrenaline brings about certain changes. There is an increase of muscle hexosephosphate with no change in the phosphocreatine or adenosinetriphosphate content coupled with a reduction of plasma phosphate and a drop of the urinary phosphate so marked that at times none can be detected [Allan *et al.*, 1924; Cori and Cori, 1931; Bell, 1932].

Another interesting observation is that creatine given by mouth to dogs causes a fall of the blood phosphate and a marked decrease or even complete absence of urinary phosphate [Brown and Imrie, 1931]. Clarkson [1932] has shown that creatine by mouth in man produces a similar diminution of phosphate excretion.

It will be seen that changes detected in the isolated muscle are of such magnitude that in the intact organism considerable changes in the composition of the blood and urine are produced.

In dealing with the febrile cycle of malaria the observation of Borchardt [1928] that the production of experimental fever is considerably modified in the absence of the suprarenal suggests that the secretion of adrenaline occurs in that part of the malarial cycle terminating in the peak. In the same period rigors or severe muscular tremors occur. There is therefore in this phase tetanic muscular contraction superimposed on adrenaline stimulation, which as shown by Cori and Cori [1933] produces a maximum deposition of hexosephosphate. The phosphocreatine broken down by muscular contraction is rapidly reconstituted but when fatigue ensues there is some reduction in its amount, the creatine escaping into the blood and being lost. This is the time at which maximum output of creatine in the urine was found. The phosphate presumably is retained as hexosephosphate. Morais [1932] has shown that in fever produced by dinitro-α-naphthol the phosphocreatine in the muscles of pigeons is reduced from an average of 29·6 mg. to an average of 5·4 mg. per 100 g. muscle.

At the end of this phase, which presumably coincides with the maximum temperature and cessation of rigors, the hexosephosphate due to tetanisation is dissociated, and since the phosphocreatine has been reduced there is ample opportunity for its reconstruction since there is a slight increase in the creatine of the blood and an increase in the available phosphate. This is probably the same type of reaction as occurs when creatine is given by mouth, *i.e.* an increased phosphocreatine formation when the materials are available. The exact time at which the blood phosphate will commence to rise is a resultant of two factors—the rate of breakdown of the hexosephosphate formed by tetanisation and the rate of re-formation of phosphocreatine. At this point there occurs therefore a fall of the blood creatine and a variable response of the blood phosphate. Later on the balance is shifted in favour of a rise in phosphate by the addition of the breakdown products of the extra hexosemonophosphate formed in response to the adrenaline liberated in the previous phase, this disappearing more slowly according to Cori and Cori [1931]. A reduction in the rate of phosphocreatine formation due to a reduction of the available creatine together with an increase in the phosphocreatine already formed is also a possible factor. It is at this point that the low values for blood and urine creatine were found.

In the early days of the malaria the phosphate of the blood is plentiful and is not completely spent by the time of the peak. All the available creatine can be used to reform phosphocreatine and so minimum values for creatine in the urine are obtained. In the later period the phosphates have decreased in amount and the available phosphate is exhausted by the time the peak is reached. At this point the urine is found to contain little or no phosphate. The amount of phosphate available is now not enough to produce so big a reduction of the creatine in the blood and urine. Why the blood phosphate falls is not clear. It is stated by many authors that the phosphates are excreted in excessive amounts in fever, but in actual fact in these cases there was no increased loss of phosphate compared with the preceding non-febrile period. In many, indeed, less phosphate was lost in the urine. No attempt to determine the loss in the faeces was made and it is therefore possible that the loss occurred there and the results of von Moraczewski [1900] lend some support to this. At the same time there was a variable but definite decrease in the food intake and thus a lessened phosphorus intake.

The increase of nitrogenous output has long been recognised and it has been observed by many [*e.g.* von Moraczewski, 1900] that the ratio P/N falls in fever indicating a retention of phosphorus produced from protein destruction. On the other hand creatinine has been found to be excreted in excess in fever, and since it seems certain that creatine is the precursor to the urinary creatinine there should be no shortage of available creatine. This is borne out by the results obtained here.

The early low creatine values in the urine and the later low phosphate values can thus be accounted for. It is not so easy to account for the simultaneous low values occurring in the days immediately following the administration of quinine and the return of the temperature to normal. These low figures differ from the preceding ones in that they coincide in time and it is only in this period that a specimen of urine was obtained containing neither detectable creatine nor phosphate. These low values did not occur throughout the whole day but occurred on the whole in the period 5 a.m. to noon. The easiest explanation is to suppose that during and at the end of the sleep period there is a regeneration of the phosphocreatine content of the muscles which in all probability were depleted during the attack.

The significance of the absence of creatine and phosphate from the urine is of interest. According to Wigglesworth and Woodrow [1923], Brain *et al.* [1928] and Walker [1932] the kidney has a threshold for phosphate about 2–3 mg. per 100 ml. calculated from the slope of the curve relating urinary and blood phosphate concentration. Walker indeed states that no example in man of reaching this hypothetical threshold has been recorded. It will be seen from Table II that the values of the blood phosphate obtained at times when no phosphate was being excreted agree well with the calculated value but show a fairly wide variation. This variation might be increased by the unavoidable use of whole blood. In the rapid fall which occurs the red cells might lag behind the plasma in regard to the phosphate content and thus misleading high values would occur. If the rate of diffusion were the limiting factor, however, equilibrium would be established almost instantaneously.

Table II. *The level of the blood phosphate in relation to the urine phosphate.*

Phosphate in urine	Blood phosphate (mg./100 ml.)						
	Case L. S.	S. S.	F. F.	E. S.	J. W.	G. R.	E. G.
None	2·8	3·2, 3·3	2·0	—	—	1·9, 1·7	2·0
Trace	3·2	4·6	—	1·8, 1·9	2·7	2·5	—

In the case of creatine the question is more complex. In the adult no creatine escapes into the urine although it appears in the blood in the same (or even greater) amounts as are found in children actually excreting creatine. Examination of the values obtained here gives no support for a rigid threshold although not enough figures are available to be dogmatic with so rapid a variation of the blood creatine.

Case E. C. Lowest value of creatine 4·5, 6·5, 4·5. Urine +
,, ,, 5·2 ,, 0
,, ,, 6·1 ,, Trace
Case E. G. ,, 4·2 ,, +

It is quite possible that all the chromogenic substance formed after hydrolysis is not creatine, or again the creatine may be in part combined with some larger molecule in its transition from muscle creatine to urinary creatinine, or as suggested by Hunter and Campbell [1918], the greater part of the creatine is in the red cells and only small amounts are present in the plasma. It is probable however that in the rapid variations in the creatine values the excess creatine is in the plasma since it is assumed that the variations are due to the muscles.

SUMMARY.

1. The phosphate, creatine and creatinine in blood and urine have been studied in cases of malaria produced in children.

2. There is a fall of the phosphate in the blood during the rise of the temperature followed by a rise starting about the time the temperature begins to fall. The fall may be so great as to cause the phosphate to disappear from the urine.

3. There is a rise of the creatine in the blood with the temperature followed by a fall as the temperature falls. The creatinine follows the same course only somewhat delayed in time. As with phosphate the fall is great enough to cause the normal creatinuria of children to disappear.

4. When the fever is stopped by giving quinine there is a simultaneous fall of both phosphate and creatine.

5. Evidence for a threshold for renal excretion of phosphate is put forward. The threshold value in these children lies between 1·8 and 3·2 mg. per 100 ml., the lower values being more probable. No evidence of a threshold for creatine could be found.

6. An explanation has been sought on the basis of a rapid breakdown of phosphocreatine and formation of hexosemonophosphate in the muscles by the combined action of adrenaline and muscular action, followed by an equally rapid reversal when the temperature peak is passed.

I wish to thank Dr D. Nabarro for permitting me to investigate the children under his care and for the helpful interest he has taken in the work. My especial thanks are due to Nurse Irving, without whose unremitting care the many specimens of urine required could never have been obtained, and to Miss Dora Ham on whose shoulders the greater part of the analytical work has fallen and who, as well, has shared in the collection of the blood at the various inconvenient hours that have been necessary. I also wish to thank Dr P. Eggleton for helpful criticism of the work.

REFERENCES.

Allan, Dickson and Markowitz (1924). *Amer. J. Physiol.* **70**, 330.
Bell (1932). *Biochem. J.* **26**, 1601.
Borchardt (1928). *Arch. exp. Path. Pharm.* **137**, 45.
Brain, Kay and Marshall (1928). *Biochem. J.* **22**, 628.
Brown and Imrie (1931). *J. Physiol.* **71**, 222.
Clarkson (1932). *J. Physiol.* **75**, P 29.
Cori and Cori (1931). *Amer. J. Physiol.* **94**, 581.
—— —— (1933). *J. Biol. Chem.* **99**, 493.
Eggleton and Eggleton (1927). *Biochem. J.* **21**, 190.
Fiske and Subbarow (1927). *Science*, **65**, 401.
Garratt (1904). *Medico-Chirurgical Trans. London*, **87**, 163.
Gemmill and Ribeiro (1933). *Amer. J. Physiol.* **103**, 367.
Havard and Reay (1926). *J. Physiol.* **61**, 35.
Haxthausen (1860). Diss. inaug. Halle (quoted by G. C. Garratt).
Hunter and Campbell (1918). *J. Biol. Chem.* **33**, 169.
Kael (1932). *Biochem. Z.* **245**, 452.
von Moraczewski (1900). *Z. klin. Med.* **39**, 44.
Morais (1932). *Compt. Rend. Soc. Biol.* **109**, 561.
Planet and Cardoso (1933). *Compt. Rend. Soc. Biol.* **112**, 1509.
Walker (1932). *J. Lab. Clin. Med.* **17**, 347.
Wigglesworth and Woodrow (1923). *Proc. Roy. Soc. Lond.* B **95**, 559.

CLX. THE CHEMISTRY OF THE WHITE ROTS OF WOOD.

IV. THE EFFECT ON WOOD SUBSTANCE OF *USTULINA VULGARIS* TUL.

By WILLIAM GEORGE CAMPBELL AND JAN WIERTELAK.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Aylesbury, Bucks.

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In connection with his studies of the parasitism of the genus *Ustulina* on the timbers of temperate regions Wilkins [1934, and in the press] identified *U. vulgaris* Tul. as the causal organism of a disease of lime (*Tilia vulgaris* Hayne). In view of the facts that this constituted the first experimental evidence of the parasitism of this particular fungus on lime wood in temperate climates and that doubt has frequently been expressed with regard to its injurious effect on trees, it seemed desirable to supplement the mycological study by determining the ultimate chemical effect of the fungus on the wood substance. This has now been carried out on material supplied by Mr W. H. Wilkins of the Mycology Laboratory, University Department of Botany, Oxford; and also on a sample of freshly felled beech wood, which is the usual host of the fungus.

EXPERIMENTAL.

(1) *Lime wood decayed by* U. vulgaris.

The material consisted of a 6-in. disc cut from the bole of the diseased tree. Three distinct zones were clearly discernible in the sample as follows: (1) Outer sound wood. (2) Intermediate "red wood", a dark brown-coloured zone. (3) Inner decayed wood. The appearance of the specimen suggested that the causal fungus was of the white rot type.

The wood from each of the three zones was air-dried to a moisture content of approximately 6 % and converted into a series of more or less regularly shaped pieces measuring approximately 4 × $1\frac{1}{4}$ × $1\frac{1}{4}$ in. The volume of each piece was determined by the method described in previous work [Campbell, 1932] and each piece was weighed after oven-drying at 105°. Accepting the outermost zone as sound wood the approximate percentage loss in weight sustained by the inner zones during decay was calculated by comparing the average ratio $\frac{\text{oven-dry weight}}{\text{air-dry volume}}$ for each group of decayed specimens with that for the group of sound specimens. Each group of specimens was now converted to sawdust and ground to pass a 60-mesh screen. In each case the portion remaining on the 80-mesh screen was reserved for analysis (Table I).

Table I. *The effect of* Ustulina vulgaris *on the chemical composition of lime wood* (Tilia vulgaris *Hayne*).

Results expressed as % by weight of oven-dry material.

		Intermediate "red wood"		Decayed wood			
				I		II	
	Sound wood	% of sample	% of sound wood	% of sample	% of sound wood	% of sample	% of sound wood
Moisture content	6·07	5·07	—	6·01	—	7·09	—
Average oven-dry wt./air-dry vol.	0·50	0·477		0·389		0·343	
Approximate loss in wt. (calculated)	—	—	4·6	—	22·2	—	31·4
Cold water-soluble	2·59	2·64	2·52	3·09	2·40	3·64	2·50
Hot water-soluble	3·63	3·71	3·54	4·56	3·56	5·85	4·01
1 % NaOH-soluble	27·03	27·66	26·39	26·68	20·76	29·45	20·20
Alcohol : benzene (1 : 2)-soluble	7·05	9·07	8·75	4·44	3·50	6·37	4·37
Cellulose	54·55	52·66	50·24	65·48	43·16	52·77	36·20
Lignin	23·74	24·21	23·10	24·21	18·84	25·55	17·53
Total methoxyl	5·75	5·36	5·11	5·71	4·44	5·62	3·86
Methoxyl in lignin	4·07	3·90	3·72	4·02	3·13	4·15	2·85
Methoxyl in lignin as % of lignin	17·13	16·10		16·61		16·24	
Total pentosans	20·41	21·76	20·76	21·84	16·99	20·17	13·84
Pentosans in cellulose	12·34	12·55	11·97	12·59	9·79	12·51	8·58
Pentosans not in cellulose	8·07	9·21	8·79	9·25	7·20	7·66	5·26

(2) *Beech wood decayed by* U. vulgaris *in pure culture.*

A sample of freshly felled beech wood (*Fagus sylvatica* L.) was converted to sawdust and air-dried. A sample of 60–80 mesh material prepared from this was sterilised by steaming at 100° for 30 minutes on each of three consecutive days, inoculated with a pure culture of *U. vulgaris* and incubated at 20°. The authors are indebted to Mr W. P. K. Findlay for carrying out the inoculation. After 14 months the decayed wood was washed free from acid with cold water, dried at 105°, weighed and analysed (Table II).

Table II. *Analysis of 60–80 mesh beech wood decayed by* Ustulina vulgaris.

Results expressed as % by weight of oven-dry sound wood.

	Sound wood	Decayed wood
Duration of decay	—	14 months
Loss in wt.	—	3·17
Cold water-soluble	0·32	0·75
Hot water-soluble	1·23	0·72
1 % NaOH-soluble	14·88	15·12
Cellulose	59·89	55·26
Lignin	21·38	21·14
Total pentosans	25·10	23·15
Pentosans in cellulose	14·95	12·55
Pentosans not in cellulose	10·15	10·60
Total methoxyl	6·48	6·28
Methoxyl in lignin	4·73	4·50

DISCUSSION.

The results in Table I indicate that in the living tree *U. vulgaris* produces a typical white rot. Both lignin and carbohydrates are attacked and the alkali-solubility, when calculated as a percentage of sound wood, decreases steadily as decay proceeds.

The results for the intermediate zone of so-called "red wood" are of considerable interest. From a visual examination it was doubtful whether the fungus had penetrated into this zone to any appreciable extent although the wood was stained a reddish brown colour, which suggested that at least part had undergone chemical change. The analytical data make it abundantly clear that the wood substance in question was in an early stage of decay. A loss in weight of approximately 4·6 % was sustained and this is almost wholly accounted for by depletion of the Cross and Bevan cellulose. The furfuraldehyde-yielding complexes associated with the cellulose only exhibit the faintest indication of decomposition. At this early stage of decay the lignin is also attacked to a slight extent. Both the methoxyl groups and the main portion of the lignin complex would appear to be involved. With regard to extractives it is noteworthy that the decayed wood contains less water- and alkali-soluble material than sound wood when the results are all expressed on the same basis. At the same time the fraction soluble in alcohol:benzene is greater in the decayed than in the sound wood. It is therefore suggested that the increment to the alcohol:benzene-soluble fraction is derived from transition products in the break-down of the wood substance. The red-brown colour of the intermediate zone may thereby be explained.

As the decay becomes more advanced the nature of the ultimate chemical changes is in all respects similar to certain other white rots which have been studied [Campbell, 1932; Wiertelak, 1932]. At the most advanced stage examined approximately one-third of the cellulose and one-quarter of the lignin were decomposed. Depletion of the furfuraldehyde-yielding complexes was also well marked. It is therefore apparent that the fungus derives the greater part of its nourishment from the carbohydrates of the cell wall substance of the wood. It is observed that as decay proceeds the solubility of the wood in alcohol: benzene exhibits a decline. This would appear to indicate that the by-products formed in the early stages of decay are ultimately decomposed.

Turning now to the data in Table II it can be seen that although the saprophytic attack of *U. vulgaris* on wood substance is slow it closely resembles, in its early stages at least, the parasitic action (Table I).

As before, attack is concentrated on the cellulose from the outset. In this case there is more definite indication of early attack on the furfuraldehyde-yielding complexes associated with the cellulose, and the attack on lignin at this stage has been confined to methoxyl groups.

SUMMARY.

Ustulina vulgaris Tul. as a parasite on lime wood (*Tilia vulgaris* Hayne) produces a type of decomposition which must be characterised as a white rot. The chief source of fungal nourishment, however, is the carbohydrate portion of the cell wall. Lignin is also decomposed but not to the same extent as the Cross and Bevan cellulose.

The saprophytic action of the fungus on beech wood, although slow, is chemically of the same order as the parasitic action.

The experimental data obtained from decayed lime wood suggest that this fungus must have a pronounced detrimental effect on the mechanical properties of the wood.

We wish to express our indebtedness to the Polish Fund of National Culture for financial assistance and to the Department of Scientific and Industrial

The precipitate from pancreatin treated with NaOH constituted about 3·1 % of the total pancreatin and could be separated into an active and an inactive fraction. The former was a denatured protein containing 13·6 % N, and the active portion could be recrystallised from alcohol to give a substance of M.P. 59°. The latter possessed the properties of a mixture of saturated fatty acids such as palmitic acid and constituted 14·7 % of the precipitate.

It was indeed found possible to prepare a mixture of fatty acid and caseinogen which reproduces the surface active properties of pancreatin. Thus a mixture of sodium oleate and caseinogen in the proportions of 1:2 by weight gave the same p_H optimum for displacement of both protein and tripalmitin films as did the same quantity of pancreatin. Further, the action of sodium fluoride on such a synthetic mixture was to cause visible flocculation of protein and with it much adsorbed fatty acid, thus accounting for the observed inhibitions of surface activity by sodium fluoride. It is worthy of note that some species of complex between the sodium oleate and caseinogen must exist in solution for the action on films of sodium oleate alone is not affected by the addition of sodium fluoride; and as already noted the fatty acids are only extractable with hot alcohol.

Specific enzymes.

In previous publications experiments have been described showing the progressive attack of monolayers of caseinogen by specific proteolytic ferments, the reaction proceeding in stages according to the particular enzyme fraction employed. The following newly available preparations have been studied.

(1) *Chymotrypsin.* This possesses negligible lipolytic and considerable proteolytic properties corresponding to a more extensive breakdown than the original crystalline trypsin. On injection beneath films of tripalmitin and of caseinogen, the latter was digested much further (a further 35 mv. as measured by the method of surface potentials) than by crystalline trypsin. The tripalmitin was scarcely attacked at all.

0·123 g. chymotrypsin dissolved in 300 ml. $M/25$ phosphate buffer at p_H 8 (25°) reduced the surface potential of a caseinogen monolayer from 280 mv. to a final constant value of 225 mv. in 15 minutes. This corresponds to the bulk activity of chymotrypsin. Treatment with NaOH or incubation with crystalline pepsin caused rapid inactivation for bulk and surface activity. Like crystalline trypsin, chymotrypsin solutions give a gelatinous rigid film at the air/liquid interface; thus one observes the caseinogen film turn from gelatinous to liquid during the reaction, while the free surface of the enzyme solution remains gelatinous. The chymotrypsin can neither digest itself nor displace the partially digested caseinogen film.

(2) *Chymotrypsinogen.* This material, from which chymotrypsin is prepared by activation with crystalline trypsin, is completely inactive for surface reactions as it is in bulk. The free surface potential value of chymotrypsinogen solutions is much higher than that of its active form.

(3) *Crystalline pepsin.* This shows no lipolytic activity either in bulk or towards tripalmitin films, but digests caseinogen films at p_H 2·0, with a reduction in the potential of the protein film from 380–315 mv. in 20 minutes, at a concentration of 0·1 ml. pepsin soln./200 ml. The same phenomenon is apparent here as in the case of chymotrypsin, namely that during the reaction the caseinogen film, initially a rigid two-dimensional gel, becomes perfectly fluid owing to digestion, while the free surface of the enzyme solution remains gelatinous throughout. Crystalline pepsin possesses no surface activity at p_H 8·0.

(4) *Enzymes separated by alumina.* Pancreatin freed from lipase by progressive adsorption with alumina *B* gave proteolytic solutions which digested caseinogen films in a manner similar to Northrop's chymotrypsin. Treatment with NaOH completely inactivated these preparations in respect to surface as well as bulk activity, and on acidifying the solution a precipitate was obtained at p_H 4·6 which on redissolving was completely inactive. Thus adsorption on alumina effects the removal of all fatty acids as well as of lipase from pancreatin.

The above specific enzyme preparations on injection beneath unimolecular films of long chain alcohols or amines gave no evidence of any complex formation.

The limitations of the surface technique in this field are now clear. It is seen that a fatty acid-protein complex can simulate the expected behaviour of a lipolytic enzyme in respect to p_H optimum and fluoride inhibition and in replacing for example a tripalmitin film by a film of fatty acid. Further, these complexes are obtained from pancreatin by processes such as boiling or alkali treatment which destroy the true enzymes. It is only specific ferments such as are obtained by less drastic treatment, *e.g.* ammonium sulphate precipitation or adsorption on alumina which can be examined with safety. It is of interest, however, that it appears to be impossible to separate the lipase fraction of pancreatin from lipoids, including fatty acids.

In regard to the protein-fatty acid complex, evidence of such adsorption complexes was obtained by du Noüy [1926] from measurements of the surface tension of mixtures of varying preparations of sodium oleate and albumin. Roughly 200 molecules of the soap can be adsorbed to a protein molecule of molecular weight about 35,000, *i.e.* about one molecule per side-chain. In the light of the present work there must be an equilibrium between such a complex and free fatty acid or soap. Thus a film of tripalmitin spread on a solution of the soap-protein or fatty acid-protein complex is replaced by a film of soap or fatty acid, according to the p_H, possessing a surface potential some 100 mv. higher than that of the soap-protein complex accumulating at the free surface. The simplest interpretation is that the close-packed film of tripalmitin cannot be penetrated by the large molecules of protein or of protein-fatty acid complex, but that the free fatty acid or soap molecules can penetrate and displace the tripalmitin over a wide range of p_H. The displacement of films of tripalmitin and of protein by fatty acid or soap alone is closely related to the surface tension as dependent on p_H. Fig. 4 shows the surface tension-time curve at various p_H values for sodium oleate as measured by the ring method. The maximum and most rapid surface tension lowering occurs around p_H 7. With increasing acidity the rate of reaching the maximum surface tension lowering decreases markedly and the effects are complicated by the formation of oleic acid emulsions with somewhat variable properties. On the alkaline side the rate of surface tension lowering increases, so that at p_H 12 the maximum value is reached instantaneously, but this maximum lowering is very much smaller, namely 7 dynes/cm. as compared with 25 dynes/cm. at p_H 7·8 for the same concentration of sodium oleate, 0·0005 %.

Now in the neighbourhood of neutrality soap displaces both tripalmitin and protein films very rapidly; at p_H 2 tripalmitin films are slowly displaced by fatty acid and protein films only very slowly. At p_H 12 the tripalmitin film is displaced very slowly and the protein films very rapidly. The inactivity in the acid solution is due to the actual emulsion formation referred to, and in the alkaline solution the explanation in the case of the displacement of protein is that the surface tension lowering of the soap solution is sufficiently great and rapid to displace the protein films, which have a still smaller surface tension

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REFERENCES.

Campbell (1932). *Biochem. J.* **26**, 1829.
Wiertelak (1932). *Bull. Acad. Polonaise des Sci. et Lett. Série B, Sc. Nat.* **1**, 19.
Wilkins (1934). *Trans. Brit. Myc. Soc.* **28**, 320.

CLXI. TRANSFORMATION OF HAEMINS INTO BILE PIGMENTS.

By RUDOLF LEMBERG.

From the Biochemical Laboratory, Cambridge.

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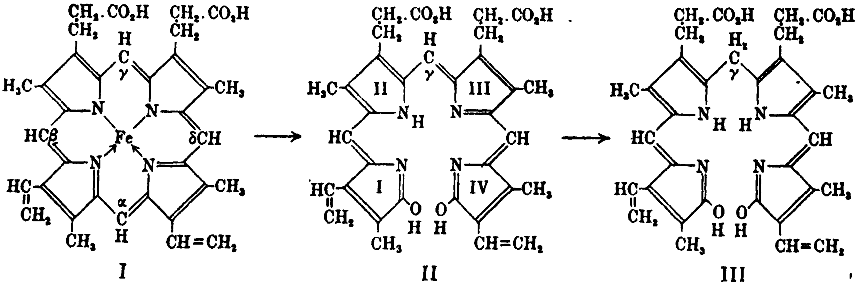
That bile pigment arises in the body by decomposition of haemoglobin is generally accepted. The work of Fischer and collaborators has confirmed the close chemical relationship between bilirubin and the colour component of haemoglobin. Hitherto nothing was known about the chemical mechanism of this transformation, nor has the formation of a bile pigment from haemoglobin or haemin *in vitro* been demonstrated with certainty. Mesobilinogen[1] has been transformed into mesoporphyrin [Fischer and Lindner, 1926, 2]; the reverse reaction however has not been accomplished. From a chemical point of view the formation of bile pigments from haemin would mean their total synthesis, since haemin has been synthesised by Fischer and Zeile [1929]. Several mesobilirubins (with a symmetrical arrangement of the side-chains) are now obtainable by direct synthesis, but the natural bile pigments which have unsymmetrically arranged and unsaturated side-chains, like bilirubin and biliverdin, cannot be synthesised in this way. A glance at the formula now established for bilirubin (III) by the investigations of Fischer since 1931 shows that it must arise from the porphyrin nucleus of haemoglobin by oxidative scission. The α CH-group is removed and replaced by two hydroxyl groups, and the porphyrin ring is thus opened. If this reaction leads to bilirubin (I → III), the γ CH-group is reduced to CH_2. Although an oxidative-reductive process is not impossible, another alternative suggests itself, *viz.* that the reaction leads at first to biliverdin [dehydrobilirubin, *cf.* Lemberg, 1934, 1] which is subsequently reduced. It has been reported by Barry and Levine [1924] that the liver reduces biliverdin to bilirubin. Moreover the bile of a number of animals (*e.g.* of the frog) contains biliverdin and not bilirubin[2].



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[1] The writer proposes to use the shorter term "mesobilinogen" instead of "mesobilirubinogen". The first name is also more logical, since mesobilinogen is the leuco-compound of mesobiliverdin *etc.* as it is of mesobilirubin.

[2] Unlike the product of autoxidation of bilirubin in alkaline solution and like uteroverdin the biliverdin of frog's bile is free from products of secondary alteration. This disproves Fischer's view that there is a genuine difference between biliverdin and dehydrobilirubin.

Several references occur in the literature to the formation of green pigments from haemoglobin or from haemin. In fact, the bile pigment nature of some of these compounds has been claimed, although the evidence has been very scanty. With the exception of Warburg and Negelein [1930] no investigator has obtained a pure substance. Moreover, the work of these authors pointed in a different direction, since they obtained substances containing iron ("green haemin").

A closer study of the literature reveals that the processes observed by the different authors leading to the formation of green pigment from haemin or haemoglobin have more in common than appears at a first glance.

From the description given by Lewin [1901] of the dichroic green-brown substance (or more likely mixture of substances), obtained from the blood of animals poisoned with phenylhydrazine and termed haemoverdin, it remains doubtful whether it belongs to the series of pigments discussed in this paper. This does not seem improbable in view of the existence of oxidative systems produced in erythrocytes by phenylhydrazine [Warburg *et al.*, 1931, 1, 2] and in consideration of the power of this compound to produce jaundice.

Parisot [1911] prepared a green pigment by incubation of haemolysed blood with adrenaline and ammonium sulphide or sodium hydrosulphite. He considered the substance to be biliverdin, since it gave the Gmelin test. This test cannot however be considered as sufficient evidence for the bile pigment nature of a substance, unless the typical absorption bands appearing at some stages of the reaction are observed. Brugsch and Pollack [1924] contested the bile pigment nature of Parisot's substance on the grounds that it did not give the diazo-test. Since biliverdin in contrast to bilirubin does not give this test, the failure of Brugsch and Pollack to obtain it is no evidence against Parisot's assumption.

Fischer and Lindner [1926, 1] observed that (proto-)haemin and also other haemins, dissolved in pyridine, were transformed at 40–50° into green pigments in the combined presence of oxygen and of reducing substances (*e.g.* polyhydric phenols, SH-compounds). Instead of these reductants yeast (which was used in the first experiments by these authors), minced plants or animal tissue (particularly liver) could be used. The authors mention the possibility that the amorphous green pigment might be a bile pigment, but their results do not harmonise in several points with this assumption. They found the green product to contain iron and to show distinct absorption bands different from those of any known bile pigment. From the description of the bands there can be no doubt that they were of the type of those given by the "green haemin" of Warburg and Negelein [1930].

By the action of molecular oxygen at 50° on pyridine-haemochromogen in the presence of hydrazine as reductant, Warburg and Negelein [1930] obtained a "green haemin" showing typical absorption bands, Warburg [1932] considered such "green haemins" as somewhat related to the green haemins of phaeophorbides and brought forward the hypothesis that the "Atmungsferment"-haemin (a green-red haemin) might stand between the red (porphyrin-)haemins and the green (chlorophyll-)haemins, the latter being oxidation products, the former reduction products of the Atmungsferment-haemin, from which they arose in the course of development. The results of the present paper show that the "green haemins" obtainable by oxidation have nothing to do with chlorophyll haemins. Warburg and Negelein did not obtain the "green haemin" itself in a pure state, but they were able to transform it by methyl alcoholic hydrochloric acid into a crystalline ester $C_{36}H_{40}O_6N_4FeCl_4$ which contained chlorine in contrast to the green haemin itself.

Schottmüller [1903] recognised that streptococci (*S. viridans*, *Pneumococcus*) produce a green pigment from haemoglobin when grown aerobically on the surface of blood agar plates or in blood broth. This process has been investigated in detail by Hart and Anderson [1933] and earlier literature on the subject has been reviewed in their paper. The pigment (which in fact is a mixture) shows in addition to other absorption bands a band in the red part of the spectrum resembling that of Warburg's green haemin. With the pigment produced by bacteria from haemoglobin Rich and Bumstead [1925] observed an atypical Gmelin reaction only. A pigment of the same properties as that produced by bacteria was prepared by Anderson and Hart [1934] by the action of non-bacterial reducing systems on haemoglobin in the presence of oxygen, and a similar mechanism was assumed to work in the formation of the pigment by bacteria.

The essential similarity of all the processes described above is that the simultaneous action of a reducing system and of oxygen on either haemochromogen or haemoglobin is necessary for the green formation.

Recently Schreuss and Carrié [1934] reported that a green pigment showing the properties of a biliverdin (it was soluble in ether and passed from it into hydrochloric acid) can be obtained in very small amounts if haemoglobin or haematin is incubated with liver "brei" or liver extract at 70° and p_H 7–8. The authors do not give any details of their method and the pigment was not isolated in the pure state. The formation of biliverdin was regarded by them as an oxidation of bilirubin during the extraction process.

It appeared particularly important to me to make a closer study of the "green haemin ester" of Warburg and Negelein, not only because it was the only pure product obtained but also for important structural implications. This ester contained four chlorine atoms and one iron atom. The results of Warburg and Negelein made it improbable that the substance was a haemin ester of a chloroporphyrin, since unlike the known tetrachloroporphyrins it could not be reduced to a normal red porphyrin. Biliverdins however form ferrichlorides, as has been shown by Fischer *et al.* [1932] and by Lemberg [1932], and it seemed not impossible that the substance in question was such a ferrichloride of a biliverdin.

EXPERIMENTAL.

For the preparation of the "green haemin" the procedure of Warburg and Negelein was used with slight alterations. It is essential to complete the transformation of haemin into green haemin as fast as possible to avoid formation of brown by-products. This could be attained more easily if only small amounts of haemin were used in each experiment; the products of several experiments were then worked up together.

In a 500 ml. wide-necked Erlenmeyer flask with almost vertical sides 0·4 g. of haemin was dissolved in a mixture of 100 ml. pyridine and 250 ml. water. This solution was kept at 50° in a water-bath and was aerated with a violent current of oxygen accompanied by vigorous mechanical stirring. A solution of 1·3 g. hydrazine sulphate in 32 ml. $N/2$ NaOH was then added. The colour changed soon to green and after 3–4 minutes the first band of haemochromogen at 557 $m\mu$ had disappeared and the spectrum of the green haemin (*cf.* below) was seen. At this point the reaction was stopped immediately and the solution cooled with ice. The filtered solutions of several successive experiments were combined and extracted without delay with ether and chloroform as described by Warburg. The black precipitate which appeared during the extraction of the brown by-products with ether included a considerable part of the "green haemin". This was extracted by an aqueous pyridine solution containing some hydrazine hydrate and some acetic acid. This part of the product however was kept separate; it yielded a considerable amount of "green haemin" but did not give crystalline ester. The main bulk of the chloroform solution was concentrated *in vacuo* at 40° to a few ml. and precipitated with light petroleum (B.P. 50–60°).

The precipitate was transformed into the "green ester" by boiling in methyl alcoholic hydrochloric acid. From 2 g. haemin 50 mg. pure crystalline ester were obtained with the following crystal properties.

Elongated platelets pointed at the ends. Extinction slightly oblique. Pleochroism: light blue-green, if long axis of platelets parallel to plane of polarisation, dark green in perpendicular orientation. Blue and green colours between crossed nicols. The substance did not melt. The solution of the compound does not show distinct absorption bands but a general absorption of the red part of the spectrum.

A few mg. of the ester were dissolved in chloroform. The dark green solution turned blue when shaken with water (colour of neutral biliverdin). The aqueous solution was found to contain ferric iron as indicated by the thiocyanate reaction. This experiment is proof that iron is not present in complex combination in the ester, but as a ferric chloride double salt.

One mg. of the ester was dissolved in 1 ml. methyl alcohol and this solution was poured into a mixture of ether and sodium tartrate solution. Biliverdin ester passed into the ether layer colouring it blue, whereas iron was found in the tartrate solution. With 1 % hydrochloric acid the pigment passed into the acid aqueous solution with green colour.

With zinc acetate and iodine biliverdins like bilirubins [Auché, 1908] are transformed into the complex zinc salts of biliviolins. Biliviolins contain two hydrogen atoms less than biliverdins [Lemberg, 1934, 2]. Their zinc complex salts are characterised by typical absorption bands and extremely strong red fluorescence. The "green ester" reacted immediately with zinc acetate to give a yellow-green solution with an absorption band in the extreme red part of the spectrum (biliverdin zinc salt); on addition of a drop of iodine the solution turned green-blue and displayed a striking red fluorescence and absorption bands at 635·0 $m\mu$ and (less strong) 582·0 $m\mu$. These bands are the same within experimental error as those of biliviolin ester zinc salt, prepared in the same way from biliverdin (634·1 and 580·8 $m\mu$) and distinctly different from those of the corresponding mesobiliviolin compound (*cf.* below). This experiment confirmed the finding that the iron is not bound in complex combination in the green ester, and made its identity with the ferrichloride of biliverdin ester very probable.

The formula of *biliverdin dimethyl ester ferrichloride* is $C_{35}H_{38}O_6N_4.HCl.FeCl_3$. (Found: C, 51·86; H, 4·80; Fe, 6·68; OCH_3, 7·60, 7·47 %. $C_{35}H_{39}O_6N_4FeCl_4$ requires C, 51·91; H, 4·86; Fe, 6·90; OCH_3, 7·67 %.) The analyses reported by Warburg and Negelein are in better agreement with this formula than with the formula $C_{36}H_{40}O_6N_4FeCl_4$ which the authors ascribed to the substance. (Found: C, 51·92, 51·79; H, 4·90, 4·82; N, 6·47, 6·46; Fe, 6·85, 7·13, 6·78, 6·64; Cl, 17·93, 17·66; OCH_3, 8·29, 7·64, 7·83 %. $C_{35}H_{39}O_6N_4FeCl_4$ requires C, 51·91; H, 4·86; N, 6·93; Fe, 6·90; Cl, 17·53; OCH_3, 7·67 %.)

Since the compound has no melting-point it was transformed into *biliverdin dimethyl ester* for identification. 40 mg. were dissolved in methyl alcohol, containing some sodium acetate, and this solution was poured into a mixture of chloroform with a dilute solution of sodium carbonate and sodium tartrate. The blue chloroform solution was washed with water, filtered, dried with Na_2SO_4 and evaporated *in vacuo*. The residue was recrystallised from methyl alcohol. It was free from iron and chlorine and melted at 208° (uncorr.). Mixing with purest biliverdin ester (M.P. 215°) gave no depression of the melting-point.

From the mother-liquor of the crude "green ester" a second small yield of crystals of the same properties was obtained. The mother-liquor of these however contained a different biliverdin which in the violin reaction with zinc acetate and iodine gave absorption bands of mesobiliviolin zinc salt (623·6, 572·2 $m\mu$). It is not however suggested that the compound in the mother-liquor was mesobiliverdin ferrichloride. The neutral iron-free ester obtained from it was easily soluble in methyl alcohol and did not crystallise completely. The following observations indicate that it might be haematobiliverdin (with two $CH(OH).CH_3$ groups).

21·39 mg. of this biliverdin were heated for 1·5 hours over P_2O_5 in a flask at 150° in a high vacuum (of the mercury pump). The substance lost 0·91 mg. (calculated loss of two mols. of water, corresponding to a transformation of two hydroxyethyl side-chains into vinyl groups: 1·19 mg.). The product had however been altered more fundamentally; it was no longer soluble in methyl alcohol, but soluble in chloroform and did not give the violin reaction. Hence a second sample was heated at 105° in the vacuum of the mercury pump. Under these conditions haematoporphyrin is transformed into protoporphyrin. After half an hour 14·54 mg. had lost no more than 0·12 mg. The heating was continued for three more hours; now the substance gave a violin reaction like biliverdin (protobiliverdin): absorption bands of the zinc salt at 632·3 and 578·7 $m\mu$. Whereas biliverdin dissolved in concentrated sulphuric acid is destroyed by heating for a quarter of an hour on a steam-bath and mesobiliverdin is not attacked at all and remains blue-green, the biliverdin of the mother liquor is changed into a blackish blue-green.

This partial alteration of the side-chains occurs in the preparation of the "green haemin" and not in the succeeding esterification, since biliverdin ferrichloride is completely stable under the conditions of the esterification, and since the amount of by-product formed varies according to the purity of the green haemin.

Preparation of mesobiliverdin ester from mesohaemin.

Since the corresponding mesobiliverdin compounds are well-characterised substances, and since it was interesting to investigate whether or not the reaction mechanism of the formation of "green haemin" is bound up with the vinyl side-chains I subjected mesohaemin to the same process as haemin above. Like haemin, mesohaemin was transformed into a green haemin and the latter into a green ester, containing iron and chlorine. The oxidation time extended for 6 minutes. From 0·8 g. mesohaemin 60 mg. of well crystallised green ester were obtained. The substance decomposed at 261° (uncorr.) under the following conditions. The paraffin-bath was brought to 100°, before the thermometer with the substance was inserted, and then the temperature slowly raised. Under the same conditions mesobiliverdin dimethyl ester ferrichloride ("ferrobilin ester" of Fischer) decomposed at 265° (uncorr.). With more rapid heating higher decomposition points are found. (Found: C, 51·44, 51·97; H, 4·99, 5·25; Fe, 6·77, 6·10; Cl, 18·42 %. $C_{35}H_{43}O_6N_4FeCl_4$ requires C, 51·66; H, 5·33; Fe, 6·87; Cl, 17·45 %.)

With zinc acetate and iodine the solution of the green ester turned blue exhibiting a striking red fluorescence and absorption bands at 623·2 and 572·3 $m\mu$ characteristic for mesobiliviolin zinc salt. Mesobiliverdin ester prepared from bilirubin showed absorption bands at 623·7 and 573·3 $m\mu$, if treated with zinc acetate and iodine[1].

40 mg. of the ferrichloride were dissolved in chloroform and freed from iron and HCl by washing with dilute sodium carbonate solution containing some tartrate. The residue of the dried evaporated chloroform solution was crystallised from methyl alcohol; 20 mg. of characteristic well-formed prisms crystallised out; M.P. 218–219° (uncorr.), no depression with mesobiliverdin dimethyl ester (M.P. 220° uncorr.). (Found: C, 67·39; H, 6·85 %. $C_{35}H_{42}O_6N_4$ requires C, 68·34; H, 6·89 %.)

From the mother-liquor of the ferrichloride 25 mg. of a biliverdin ester (according to the violin reaction of the meso-type) were prepared which did not crystallise as well, were more easily soluble in methyl alcohol and had a lower M.P. (179–180°). The ferrichloride of this ester was easily soluble in methyl alcohol in contrast to that of mesobiliverdin ester. From these observations it can be concluded that the product is not impure mesobiliverdin ester, but consists of a different biliverdin ester.

Verdohaemochromogen ("green haemin") and mesoverdohaemochromogen.

In contradistinction to the green esters which have now been recognised as ferrichlorides of biliverdins, the green haemins contain the iron still in complex combination. With zinc acetate and iodine they do not give the violin reaction. The iron cannot be removed by washing with water or with alkali. From the results of the investigation to be described below it will be seen that they are pyridine-haemochromogens. I propose to name the "green haemin" of Warburg verdohaemochromogen and the corresponding compound from mesohaemin mesoverdohaemochromogen.

The two compounds are very similar, but they can be distinguished from one another by the position of their absorption bands, those of the meso-compound lying nearer to the ultra-violet. This difference in the situation of the bands of proto- and meso-compounds is also true for the porphyrins, haemochromogens and biliviolins. Verdohaemochromogen dissolved in pyridine-water shows a sharp absorption band in the red with its centre at 662 $m\mu$ and two weaker bands in the green (533·1 and 500·8 $m\mu$). Mesoverdohaemochromogen under the same conditions shows the bands 644·2, 525·4 and 494·4 $m\mu$. In chloroform (containing pyridine) the first two bands lie more towards the infra-red, whereas the third has the same position as in water (verdohaemochromogen 663·5, 535·7 and 500·8 $m\mu$, mesoverdohaemochromogen 650·7, 527·8 and 495·1 $m\mu$).

If verdohaemochromogen in an aqueous solution containing much pyridine is shaken with air the type of the spectrum is little changed. The bands become less sharp, particularly that in the red, which becomes broader and is shifted towards the infra-red. Mesoverdohaemochromogen behaves in the same way. These aerated solutions contain parahaematins and have a bluish green colour rather than the yellowish green colour of verdohaemochromogen solutions. Parahaematins are also obtained if the verdohaemochromogens precipitated from the chloroform solution by light petroleum have stood some time exposed to the air. The fresh precipitate however consists, at least to a large extent, of the haemochromogen, as will be seen below. Verdohaemochromogens are evidently more stable towards atmospheric oxygen than haemochromogens.

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[1] The situation of the absorption bands depends somewhat on the purity of the violin, admixture of much verdin causing a shift of the bands towards the infra-red. It depends also on the p_H of the mixture, alkali shifting the bands towards infra-red. The difference in the position of the biliviolin and mesobiliviolin bands is however so great that compounds of the proto and of the meso series can be clearly distinguished in this way.

If the verdohaemochromogens are shaken with air in a solution containing no great excess of pyridine, the absorption bands disappear and are replaced by diffuse absorption at both ends of the spectrum. On addition of sodium hydrosulphite the olive solution turns green and displays the bands of the haemochromogens (in the case of verdohaemochromogen it is essential to use only small amounts of reductant). We can conclude from this experiment that the affinity of verdohaematin for pyridine is much less than that of verdohaem. Pyridine-verdoparahaematin which is formed by the oxidation of the verdohaemochromogen decomposes in water into verdohaematin and pyridine, unless a great excess of pyridine is present. By $Na_2S_2O_4$ verdohaematin is reduced to verdohaem, and the latter, owing to its greater affinity for pyridine, recombines with it. This is a complete parallel with the behaviour of haematin and haem towards pyridine.

If sodium hydrosulphite is added to a solution of "green haemin" in pyridine-water the solution turns yellow. The two absorption bands in the green disappear and are replaced by general absorption in the blue part of the spectrum, whereas the band in the red remains but is shifted towards the ultra-violet (652 $m\mu$). On shaking with air the solution turns green again, the bands in the green reappear and that in the red returns to its previous place. A ready explanation for this reversible reduction with sodium hydrosulphite would be that the green colour and the bands in the green were properties of a parahaematin compound which is reduced by sodium hydrosulphite to the yellow haemochromogen. The "green haemin" would then be a parahaematin and not a haemochromogen. This is, however, not in accordance with the experiments described above and it can be proved that such an explanation is incorrect. The reaction with hydrosulphite is given by verdohaemochromogen only, mesoverdohaemochromogen remains green with hydrosulphite and the absorption bands in the green do not disappear. This shows that the unsaturated side-chains of the proto-compound are somehow involved in this reaction.

The behaviour of the "green haemin" towards alkali also gives evidence that the absorption bands in the green belong to the haemochromogen. If a solution of sodium carbonate is added to a solution of "green haemin" in pyridine-water the absorption bands disappear slowly and the olive-green solution obtained shows diffuse absorption in the red and blue parts of the spectrum. If now a small amount of hydrosulphite is added the solution turns green and displays the spectrum with the three bands of the green haemin; if more hydrosulphite is added the solution becomes yellow and the bands in the green disappear. This experiment can only be explained in the following way. Pyridine-verdoparahaematin, which arises by oxidation of verdohaemochromogen in atmospheric oxygen, is unstable in alkali: thus it dissociates into verdohaematin and pyridine. By the reduction verdohaem is formed which unites with pyridine to give verdohaemochromogen, which is stable towards alkali. Since we obtain the absorption bands in the green in this experiment by reduction with hydrosulphite these bands must be those of the haemochromogen, and the further reversible reduction must be explained in a different way. Here again we find the verdohaem compounds behaving like normal haem compounds: haemochromogens are stable towards alkali, parahaematins are not [Keilin, 1926].

It can be shown in this way that the green haemin precipitated from the concentrated chloroform solution with light petroleum still contains verdohaemochromogen. A few mg. of the freshly precipitated "green haemin" were dissolved in 2 ml. pyridine-water and put into a Thunberg tube the stopper of which contained 1 ml. 10 % sodium carbonate solution. The tube was at once repeatedly evacuated and filled with nitrogen. The alkali was then allowed to run into the solution. The colour turned slightly more olive, indicating that a part of the haemochromogen had been oxidised and split, but the three bands were still strongly visible. The pyridine used in this experiment did not reduce haemin to haemochromogen (as impure pyridine does). When the experiment was repeated with a preparation of "green haemin" which had been kept for some hours in contact with the air, the bands disappeared after the addition of the alkali.

The pyridine-free *verdohaematin* was obtained in the following way. A few mg. of green haemin were ground with water in a mortar and left in suspension for one hour. By the filtration of this suspension a black powder was obtained which was washed several times with water. Its solution in methyl alcohol showed only one diffuse absorption band at 700–680 $m\mu$, that of mesoverdohaematin a band at 690–655 $m\mu$ (in a Zeiss pocket spectroscope). Verdohaematin was dissolved in phosphate buffer p_H 7·5 giving an olive solution without distinct absorption bands. With hydrosulphite the solution became olive-green, but no distinct bands were seen (verdohaem). If CO were passed into this solution, it changed to olive-brown and displayed distinct bands at 665 and 610 $m\mu$ verdohaem-CO). Experiments with pyridine confirmed the results obtained above. A small amount of pyridine did not produce any alteration before reduction, a great excess of pyridine gave rise to bluish green parahaematin solutions with a rather diffuse band in the red and more distinct bands in the green. Unexpected however, was the behaviour towards ammonia. After addition of ammonia $Na_2S_2O_4$ did not give ammonia-verdohaemochromogen, as had been expected, but a brown ammonia-haemochromogen with a completely different spectrum, showing one absorption band at 567 $m\mu$. The same compound was obtained by direct action of ammonia on verdohaemochromogen. Pyridine now produced a dirty yellow solution with a reddish tint in artificial light and a sharp absorption band at 565·7 $m\mu$. A closer study of this interesting reaction has yet to be made. With the exception of its behaviour towards hydrosulphite mesoverdohaemochromogen behaved similarly in every respect.

These experiments prove that the "green haemins" are haemochromogens, containing bivalent iron in complex combination and pyridine bound to the iron atom. It is only by treatment with acid that the iron is removed from this combination. Since haemochromogens are more easily split by acids into the iron-free compounds and iron salt than the compounds with tervalent iron like haemin, the easy removal of the iron from the green haemins is understandable. Unlike haemins, however, verdohaematins are also unstable towards acids and apparently even more liable to alterations than the verdohaemochromogens. Verdohaematin is easily transformed by dilute hydrochloric acid in methyl alcoholic solution into a mixture of biliverdin and some biliviolin.

Verdohaem compounds as bile pigment complex salts.

The evidence thus far brought forward has not yet excluded with certainty the possibility that in the "green haemins" the porphyrin ring was still closed, and that the scission occurred only during the preparation of the "green esters". It was not quite impossible, although improbable, that the ring of the green haemins was closed by a CO-group instead of a CH-group, and that the CO-group was replaced by two OH-groups under the hydrolytic action of the acid.

The reduction of haemins by sodium amalgam leads to porphyrinogens which on reoxidation by atmospheric oxygen yield porphyrins. The reduction of verdohaematin, however, leads to mesobilinogen which is oxidised by atmospheric oxygen to a urobilinoid pigment. 50 mg. of verdohaematin were dissolved in 1 % sodium carbonate solution and shaken with sodium amalgam for 2 hours. The slightly yellow solution was separated from the mercury and extracted with ether containing glacial acetic acid. The ethereal solution gave a strong reaction with Ehrlich's aldehyde with absorption bands at 562 and 495 $m\mu$. With zinc acetate and iodine a strong "urobilin" reaction was obtained. On standing the colourless ethereal solution gradually turned yellow, containing now a typical urobilinoid pigment. The absorption band of the zinc salt was situated at 508·6 $m\mu$, that of the acid aqueous solution of the pigment itself at 494·5 $m\mu$. This is exactly the behaviour of mesobilinogen. I have recently shown [1934, 3] that mesobilinogen, unlike urobilinogen, is oxidised by ferric chloride to a mixture containing biliverdins and biliviolins. The bilinogen obtained from green haemin behaves in this reaction like mesobilinogen and yields a biliviolin which according to the spectrum of its zinc salt is mesobiliviolin. There can be little doubt that the substance in question is mesobilinogen. When haemin was treated in the same way, the ether solution of the chromogen gave only a very feeble Ehrlich reaction. After some standing the spectrum of protoporphyrin appeared. In the aqueous solution a small amount of a yellow substance was present which gave a green fluorescence with zinc acetate, the typical shift of the absorption band towards the red, characteristic for urobilinoid pigments, was not however observed. Like verdohaematin "green haemin" itself was reduced to mesobilinogen by sodium amalgam.

This experiment proves the presence of an open bile pigment chain in the "green haemins" which is maintained in ring form by the central iron atom. The following experiments give additional evidence that the iron-free part of the green haemin is a bile pigment closely related to biliverdin.

When copper acetate was added to a solution of mesoverdohaemochromogen in a mixture of methyl alcohol and pyridine the solution turned at first yellow-green. The two absorption bands in the green had disappeared and that in the red had been shifted towards infra-red (to about 680 $m\mu$). On standing the band in the red gets weaker and is replaced by two bands at 632 and at 578 $m\mu$. The latter spectrum is that of a copper complex salt of mesobiliviolin, the former that of mesobiliverdin-copper which is oxidised by bivalent copper to the latter [*cf.* Lemberg, 1934, 1]. Mesoverdohaematin treated with copper acetate yields the same products.

It has been said above that the verdohaemochromogens do not react with zinc acetate. When however the solution is kept exposed to the air after the addition of the zinc salt, the red fluorescence and the spectrum of the biliviolin zinc salt appear slowly. Even the replacement of iron by another metal is not necessary to form complex salts of biliviolins from green haemins. When a solution of mesoverdohaemochromogen in methyl alcohol containing a little ammonia was shaken with air an absorption band at 633·7 $m\mu$ was observed.

Since iron complex salts of biliverdins and biliviolins have not yet been described[1], I investigated the action of ferrous sulphate on mesobiliverdin in ammoniacal solution. The filtrate was olive-green and showed diffuse absorption in the red and blue. After addition of pyridine and sodium hydrosulphite the green solution displayed absorption bands at 627·2 and 576·4 $m\mu$. This behaviour does not correspond with that of mesoverdohaemochromogen but with that of a haemochromogen of mesobiliviolin. Shaking the solution with air produced a series of changes which have not yet been investigated in detail. Since bivalent copper oxidises the copper complex salt of mesobiliverdin to that of mesobiliviolin, it was easily understandable that the tervalent iron formed in the presence of oxygen would oxidise the mesobiliverdin iron salt to that of mesobiliviolin as well. For this reason I tried to introduce iron into mesobiliverdin by means of ferrous sulphate in ammoniacal solution in the absence of oxygen.

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[1] The "ferrobilins" are not complex salts in this sense, but ferrichlorides.

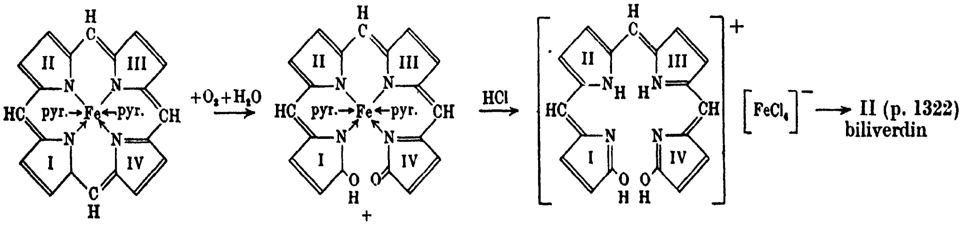
For this purpose an apparatus was used consisting of a tube containing a filter plate of porous glass with a tap below it; a small dropping funnel was fitted into the top of the tube, and the bottom was inserted into a small receiver with a tap in the side arm. Each of the three vessels was connected by means of three-way taps with the vacuum pump and with a nitrogen apparatus. Nitrogen, freed from oxygen by passage over heated copper, passed at first from the receiver, containing a little pyridine with some hydrazine hydrate as reductant, through the tap and the porous glass plate into the lumen of the tube containing mesobiliverdin in ammoniacal solution. To this solution ferrous sulphate solution, freed from oxygen by evacuation and saturation with nitrogen, was added from the dropping funnel, and after a few minutes the reaction mixture was sucked through the filter into the receiver. The two taps were then closed and the spectrum of the solution investigated. Under these conditions no complex salt is formed. The blue-green solution showed the general absorption of the red part of the spectrum characteristic of mesobiliverdin. Under the conditions of the experiment any complex iron salt formed would yield haemochromogen, the sharp absorption bands of which would not escape notice. As long as the solution was kept in nitrogen, no change was observed. When however air was allowed to enter the flask, the spectrum of biliviolin complex salt described above appeared. When hydrochloric acid was now added to this solution, mesobiliverdin ferrichloride was precipitated, leaving a blue-violet solution of mesobiliviolin (spectrum in acid solution: absorption bands with centres at 592·6 and 551·5$m\mu$, rather diffuse and connected by a shadow; main absorption band of the zinc salt: 624·9 $m\mu$). Mesobiliverdin itself apparently does not form haem compounds.

From these experiments it must be concluded that the "green haemins" are haemochromogens of bile pigments with an open ring. The iron-free compound is probably not identical with biliverdin, but unstable and easily transformed into biliverdin.

Discussion.

The reaction leading from haemins (or more exactly from pyridine-haemochromogens) to "green haemins" and from these to biliverdins can now be formulated as follows:

(The side-chains have been omitted.)



(pyr. = pyridine)
haemochromogen

verdohaemochromogen
("green haemin")

biliverdin ferrichloride

The first step leads from pyridine-haemochromogen by an oxidative process to verdohaemochromogen. The central part of the molecule remains unaltered, but the porphyrin ring is split open and transformed into a bile pigment nucleus. The usual way of writing the bile pigment formula in a straight chain is only a conventional mode of expression. Since there is free rotation around the single bonds combining pyrrole nuclei with carbon atoms the molecules of bile pigments may assume different shapes in space, *e.g.* that of a plane opened ring.

The identification of the "green esters" with derivatives of natural bilirubin (bilirubin IXα according to Fischer's nomenclature) proves that the scission occurs in the same place (α CH-group) as in the transformation of haemoglobin into bilirubin. It must be conceded that the yield of well-crystallised biliverdin ester is rather small and that isomerides with different position of the side-chains, resulting from scission of the ring at other CH-groups, might be present in the mother-liquor, but this applies also to natural bilirubin, a small fraction of which only is obtainable completely pure.

The ring having been opened at one point the stability of the complex salt becomes remarkably decreased. The central iron in its complex combination still holds the ring together, particularly as long as its six coordination places are filled, as they are in the haemochromogens. In contrast with normal haematins the verdohaematins are very unstable compounds.

The formula given above characterises the verdohaem compounds as iron salts of *iso*biliverdins. The latter are unstable and, if set free, are transformed into biliverdins (*cf.* formula II, p. 1322) by a migration of one hydrogen atom from the nitrogen of the pyrrole nucleus III to the α CO-group of ring IV.

The reaction of verdohaemochromogen with hydrochloric acid consists of the following stages. The pyridine is removed and the verdohaem oxidised to verdohaemin, this is now split into ferric chloride and *iso*biliverdin which rearranges to give biliverdin. This isomerisation transforms the nitrogen in ring III into a basic tertiary nitrogen which adds a proton. There is in addition esterification of the two carboxyl groups in the side-chains by the methyl alcohol. $FeCl_4^-$ ions are always present in solutions of ferric chloride and since the ferrichloride is the least soluble salt, it crystallises out.

The fact that isomerisation of biliverdin is necessary for the formation of "green haemin" explains why this cannot be accomplished from biliverdin with ferrous salt. The zinc and copper compounds of biliverdin are probably complex salts of biliverdin itself and not of *iso*biliverdin, since unlike the iron salt they are obtained from biliverdin, and since they show an absorption spectrum very different from that of verdohaemochromogen. Both types of complex salts can however be transformed by oxidation into complex salts of biliviolins[1].

The transformation of haemin into biliverdin is also of interest for the question of the nature of the side-chains of bilirubin. It suggests that bilirubin and biliverdin contain two vinyl groups like haemin. Fischer has often discussed this question and has repeatedly altered his views. Quite recently Fischer and Haberland [1935] have come to the conclusion that bilirubin contains one vinyl group, whereas the second unsaturated side-chain is present in the form of a dihydrofuran nucleus (*cf.* formula on p. 1333). Although it is not quite impossible that such a ring is formed during the reaction leading from haemin to biliverdin by closing a ring between the vinyl group in position β and the hydroxyl group in position α at ring IV, it does not seem probable.

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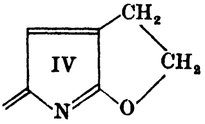
[1] H. Fischer who had previously confused the blue colour of the neutral biliverdins with the similar colour (although very different spectrum) of the acid biliviolins and assumed that the latter were isomerides of the former, has now [Fischer and Haberland, 1935] recognised his mistake and has arrived at the formula for biliviolins which I proposed in my paper before the Biochemical Society on November 16th, 1934, and which has been published in a short note [1934, 2]. Fischer has overlooked this note.



Moreover, the evidence brought forward by Fischer for the existence of this ring is unconvincing. It is based (1) on the fact that the behaviour of the unsaturated groups in bilirubin towards reagents is different from that of these groups in haemin; (2) on the isolation of a substance assumed to be a pyrrofuran compound, obtained in small yield by the action of nitrous acid on bilirubin; and (3) on the isolation of a dihydrobilirubin with one vinyl group and one ethyl group by catalytic hydrogenation of bilirubin.

As regards the first point, these differences concern both unsaturated groups in the same way; since only one can be present in form of a dihydrofuran ring (on account of the position of the unsaturated side-chain in ring I relative to the hydroxyl group), the observed differences of the bilirubin side-chains from those of haemin must be explained in a different way.

The existence of the pyrrofuran compound is very doubtful. In later experiments Fischer [1915] tried in vain to reproduce this substance, nor was Dr Mühlbauer (unpublished observation) able in my laboratory to obtain a substance of the properties described in the first paper by Fischer and Hahn [1914]. In a short note Fischer and Hess [1931] mentioned that the substance in question reacted with phenylhydrazine and possibly was methylhydroxyvinylmaleinimide (although this substance would yield analytical values quite different from those obtained by Fischer and Hahn). In spite of this uncertainty the substance is now again used as an argument in favour of the existence of a dihydrofuran ring. Previously Fischer had assumed a furan ring, although this was in evident disagreement with the amount of hydrogen necessary for the transformation of bilirubin into mesobilirubin and mesobilinogen.

The third argument of Fischer is the fact that one of the unsaturated groups of bilirubin is more easily reduced to an ethyl group by catalytic hydrogenation. The partially hydrogenated substance has an ethyl group in ring IV and a vinyl group in ring I. Provided that Fischer's hypothesis is correct, it would be necessary to assume that by catalytic hydrogenation the dihydrofuran ring is split open to ethyl and hydroxyl more readily than the vinyl group is saturated. This is very improbable, and Fischer's results are more easily explained by the simple assumption that the vinyl group in the neighbourhood of the α-hydroxyl reacts more rapidly. For these reasons it is suggested that bilirubin contains two vinyl groups like haemin.

The following observation may throw light upon the question why the vinyl group in the neighbourhood of the hydroxyl group[1] reacts more rapidly with catalytically activated hydrogen. Verdohaemochromogen is reversibly reduced by $Na_2S_2O_4$; since mesoverdohaemochromogen is not reduced, the unsaturated side-chain must play a rôle in this reversible reduction, which cannot involve the iron, since this is already in the bivalent form before the reduction. The yellow haemochromogen, obtained by reduction from verdohaemochromogen and reoxidisable to it, is probably a monohydroverdohaemochromogen in which one hydrogen atom is added to the vinyl group in ring IV. This radical is stabilised by the proximity of the α CO-group.

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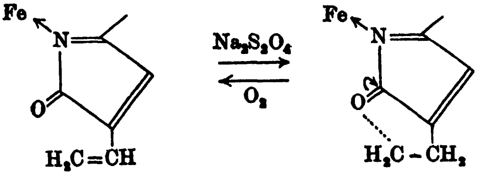
[1] Of the lactim form (—C(OH)=N—) or the CO-group of the lactam form (—CO—NH—) of bilirubin, which are probably in equilibrium.



(It is hardly necessary to mention that the cyclic structure suggested for the radical has nothing in common with a dihydrofuran ring, the linkage of the side-chain with the oxygen being a single electron.) This double oxidation-reduction system of verdohaemochromogen (in respect of the iron atom and the unsaturated-side chain) might be of interest with regard to the possible catalytic activities of the substance.

The mechanism of the reaction leading from haemin to verdohaemochromogen remains still to be investigated. Undoubtedly it is the haemochromogen which is oxidised, but whether oxygen performs this directly, or the tervalent iron of haematin, or whether oxygen and haematin are necessary for the reaction cannot yet be decided.

The action of H_2O_2 may be taken into consideration. McLeod and Gordon [1922] and Hart and Anderson [1933] have arrived at different conclusions with regard to the rôle played by H_2O_2 in the formation of the "green pigment" from haemoglobin. In this respect it is important to mention that other greenish haematins exist, and that they are present in the "green pigment" obtained from haemoglobin[1]. No definite conclusions as to the reaction mechanism of the formation of verdohaemochromogen can thus be drawn from the investigations of the different authors on the formation of "green pigment" from haemoglobin. Verdohaemochromogens are easily destroyed by H_2O_2 even in very dilute solution. The possibility that H_2O_2 in extremely small concentrations is the active agent, cannot be quite excluded, but it seems more probable that the formation of an unstable iron-peroxide of the haemochromogen itself may be the first step followed by transference of the oxygen to the porphyrin nucleus; or the reaction may be of a more complicated nature and require the presence of haematin compound with tervalent iron together with haemochromogen; the former may either oxidise haemochromogen directly or by the way of a chain reaction. However that may be, it is certain that the reaction is essentially a process in which the catalytic activity of the haem compound is applied to its own molecule.

It is suggested that the formation of bile pigment in the body may be caused by a similar process. The system of Warburg and Negelein studied above is of course only a model of the system responsible for bile pigment formation in the body. It differs in that the compound oxidised in the body is haemoglobin and not pyridine-haemochromogen, so that the resulting verdohaem compound would be either verdohaemoglobin or globin-verdohaemochromogen. Instead of hydrazine we have reducing systems. It is unnecessary to assume the action of a special enzyme for the oxidation of haemoglobin, the haem of the molecule itself or of cytochrome working as catalyst. Enzymes may play a rôle, however, in the process in so far as they provide the reducing systems [*cf.* Hart and Anderson, 1933; Anderson and Hart, 1934]. The transformation of the verdohaem compound into biliverdin cannot be brought about in the animal body by any of the reactions described above; here further research is necessary. It is evident however from the ease with which this transformation proceeds that its occurrence under physiological conditions is likely.

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[1] I am indebted to Anderson and Hart for sending me samples of green pigment produced by pneumococcus and by non-bacterial reducing systems from haemoglobin. In confirmation of the spectroscopic investigation published in their paper I found that both pigments give the same absorption spectrum in pyridine solution with hydrosulphite. The spectrum is that of a mixture of haemochromogens, *i.e.* of a good deal of unaltered protohaemochromogen, of a haemochromogen with an absorption band at 620 mμ, and of a rather small amount of verdohaemochromogen. The second haemochromogen belongs to a haematin type which can be produced by autoxidation of haematin in alkaline solution (without reductant) and which is also of greenish colour ("cryptohaemin" of Negelein).

Apart from these differences the essential process is probably the same. Hitherto only teleological explanations have been offered for the formation of bile pigments from haemoglobin, such as the unconvincing hypothesis of Asher [1924] that the decomposition of haemoglobin is necessary for providing the cells with Atmungsferment-iron. In consideration of the results brought forward in this paper the formation of bile pigments in the animal body is the natural consequence of the catalytic activity of the haem iron working on its own molecule. A similar explanation probably holds for the complexity of the cytochrome spectrum [Keilin, 1926].

From the physiological point of view a great deal remains to be done, but it is believed that the model now worked out will provide a good guide to further research.

SUMMARY.

"Green haemin" is prepared according to Warburg and Negelein by action of oxygen on pyridine-haemochromogen in presence of hydrazine. A corresponding compound is obtained from mesohaemin.

The "green haemins" are characterised as pyridine-haemochromogens of *iso*biliverdins. The name "verdohaemochromogens" is proposed. Their formation is due to oxidative scission of the porphyrin nucleus of the haemochromogen, catalysed by haem iron.

The "green ester", obtained by Warburg and Negelein by action of methyl alcoholic hydrochloric acid on green haemin, is identified with the ferrichloride of biliverdin ester. From mesohaemin the ferrichloride of mesobiliverdin is obtained in the same way.

The ferrichlorides can be transformed into the biliverdin esters and these can be identified with products prepared from bilirubin of gall stones. Since haemin has been synthesised, its transformation into biliverdin means the total synthesis of the latter.

It is suggested that the formation of bile pigments in the animal body proceeds in a similar way yielding biliverdin (dehydrobilirubin) which is afterwards reduced to bilirubin.

The nature of the unsaturated side-chains of bilirubin and biliverdin is discussed and it is suggested that they are vinyl groups like those of haemin.

REFERENCES.

Anderson and Hart (1934). *J. Path. Bact.* **39,** 465.
Asher (1924). Funktionen der Leber. Ärztl. Fortbildungskurse. (G. Fischer, Jena.)
Auché (1908). *Compt. Rend. Soc. Biol.* **64,** 279, 299.
Barry and Levine (1924). *J. Biol. Chem.* **59,** lii.
Brugsch and Pollack (1924). *Biochem. Z.* **147,** 253.
Fischer (1915). *Z. Biol.* **65,** 163.
—— Baumgartner and Hess (1932). *Z. physiol. Chem.* **206,** 201.
—— and Haberland (1935). *Z. physiol. Chem.* **232,** 236.
—— and Hahn (1914). *Z. physiol. Chem.* **91,** 174.
—— and Hess (1931). *Z. physiol. Chem.* **194,** 193.
—— and Lindner (1926, 1). *Z. physiol. Chem.* **153,** 54.
—— —— (1926, 2). *Z. physiol. Chem.* **161,** 1.
—— and Zeile (1929). *Liebig's Ann.* **468,** 98.
Hart and Anderson (1933). *J. Path. Bact.* **37,** 91.
Keilin (1926). *Proc. Roy. Soc. Lond.* B **100,** 129.
Lemberg (1932). *Liebig's Ann.* **499,** 25.
—— (1934, 1). *Biochem. J.* **28,** 978.
—— (1934, 2). *Chem. and Ind.* **53,** 1024.
—— (1934, 3). *Nature,* **134,** 422.
Lewin (1901). *Compt. Rend. Acad. Sci.* **133,** 599.
McLeod and Gordon (1922). *Biochem. J.* **16,** 499.
Parisot (1911). *Compt. Rend. Acad. Sci.* **153,** 1518.
Rich and Bumstead (1925). *Johns Hopkins Hosp. Bull.* **36,** 225.
Schottmüller (1903). *Münch. Med. Woch.* **50,** 849, 909.
Schreuss and Carrié (1934). *Klin. Woch.* **13,** 1670.
Warburg (1932). *Z. angew. Chem.* **45,** 1.
—— and Negelein (1930). *Ber. deutsch. chem. Ges.* **63,** 1816.
—— Kubowitz and Christian (1931, 1). *Biochem. Z.* **233,** 240.
—— —— —— (1931, 2). *Biochem. Z.* **242,** 170.

CLXII. THE TECHNIQUE TO BE SELECTED FOR DETERMINATION OF BILIRUBIN IN BLOOD BY THE DIAZO-METHOD.

BY EMANUEL GERARD GODFRIED.

From the Laboratory of Chemical Pathology, St Bartholomew's Hospital, London.

(Received April 27th, 1935.)

IN the present communication an attempt is made to decide which is the most practical method for estimating the total bilirubin in serum or plasma. To-day there are several modifications of Van den Bergh's test in use in different laboratories, and these different techniques give results which may vary quite widely, so that the findings reported by one laboratory are not comparable with those from another. Van den Bergh's direct reaction and its particular problems will not be considered now; the estimation of all the bilirubin involving treatment of the serum with alcohol, or, in other words, the "indirect quantitative test" alone will be studied.

In Van den Bergh's original description 1 volume of plasma or serum and 2 volumes of absolute alcohol are mixed and centrifuged. To 1 ml. of supernatant fluid 0·25 ml. of diazo-reagent and 0·5 ml. of absolute alcohol are added. The dilution factor is 5. The colour is compared with a standard solution of ferric thiocyanate in ether. Later on this ethereal standard was replaced by 2 % anhydrous cobaltous sulphate in water.

It was then shown that in some sera bilirubin is lost by adsorption on the protein precipitated by alcohol; this is largely prevented if the bilirubin is converted into azobilirubin before the precipitation of the proteins by alcohol, because azobilirubin is less strongly adsorbed than bilirubin itself; even more azobilirubin may be kept in solution in the alcohol if saturated ammonium sulphate is also added [Thannhauser and Andersen, 1921].

At this stage McNee and Keefer [1925] summarised the technique which might be universally adopted as follows. To 1 ml. of serum (or plasma) 0·5 ml. of diazo-reagent is added. After the maximum colour has developed 2·5 ml. of absolute alcohol and 1 ml. of saturated ammonium sulphate are added. The contents are mixed and centrifuged. The dilution factor is 4. The colour is compared with a standard solution of cobaltous sulphate (2·161 %).

Hunter [1930] then suggested that ammonium sulphate was unnecessary, and that the cobalt standard should be altered to 1·9 %, thus increasing the bilirubin estimates by some 14 %. His method simply consists in adding 0·5 ml. of diazo-reagent to 1 ml. of serum or plasma. After a short interval 2·8 ml. of absolute alcohol are added and the mixture is centrifuged, the dilution factor being about 4.

White [1932] dissolves 1·3 g. of anhydrous cobaltous sulphate in 50 ml. of distilled water, adds 40 ml. of conc. HCl (sp. gr. 1·19), mixes and cools. The volume is made up to 100 ml. and the mixture is ready for use in 24 hours.

It is more or less generally accepted that the aqueous cobalt standard is more satisfactory than the ethereal ferric thiocyanate standard; also that more accurate results are obtained if the bilirubin is azotised before the addition of alcohol. This is again clearly demonstrated by the results reported in the table below; if alcohol is added before the diazo-reagent (original method, column 2 of table) the results are nearly always lower, and may be very seriously low as compared with those where azotisation precedes the addition of alcohol (Thannhauser and Andersen's method, column 3 of table); Hunter's method gives results (column 5 of table) which are either the same as those yielded by Thannhauser and Andersen's method, or a little lower (significantly higher on one occasion only in this series), provided that the same standard is used for the two methods. Naturally the results (column 4 of table) are higher with the more dilute standard (1·9 %) recommended by Hunter. Those findings in column 5 which are a little lower than those in column 3 may be accounted for by the omission of ammonium sulphate in Hunter's modification.

No.	Original method. Cobalt standard 2·161 %	Thannhauser-Andersen method. Cobalt standard 2·161 %	Hunter's method. Cobalt standard 1·9 %	Hunter's method. Cobalt standard 2·161 %
1	1·0	6·0	11·4	10·0
2	0·5	1·2	1·8	1·6
3	4·0	14·0	16·0	14·0
4	2·5	6·4	7·3	6·4
5	0·7	2·8	2·3	2·0
6	12·0	18·0	17·0	15·0
7	—	8·0	7·4	6·5
8	20·0	25·0	27·4	24·0
9	2·0	6·0	6·8	6·0
10	2·0	6·4	7·3	6·4
11	0·7	1·8	1·7	1·5
12	—	6·0	6·3	5·5
13	—	12·0	11·5	10·0
14	—	5·8	5·7	5·0
15	—	4·5	5·1	4·5
16	—	8·5	8·2	7·2
17	0·6	2·0	2·1	1·8
18	—	22·0	20·5	18·0
19	—	8·4	9·6	8·4
20	1·2	1·2	1·4	1·2
21	2·0	2·1	2·4	2·1
22	1·0	1·2	1·4	1·2
23	1·5	2·4	2·7	2·4
24	0·5	3·5	4·1	3·6
25	0·8	2·1	2·4	2·1
26	0·7	1·0	1·1	1·0
27	0·6	0·8	0·8	0·7
28	0·7	1·8	2·1	1·8
29	0·8	1·3	1·6	1·4
30	1·3	2·2	—	—
31	0·6	2·0	2·1	1·8
32	4·2	4·2	4·1	3·6

All values are in Van den Bergh units. The first 19 cases showed a "direct positive reaction", *i.e.* a red colour developed within a few minutes after the addition of diazo-solution to the plasma.

White's standard again gives different results. A few comparisons were made, but the standard shows no advantage.

The following table indicates that when the same final solution of azobilirubin is compared with the 2·161 % cobalt standard and with White's standard the latter will give a result some 25 % higher than the former.

Cobalt standard 2·161 %	White's standard	Cobalt standard 2·161 %	White's standard	Cobalt standard 2·161 %	White's standard
2·0	2·5	8·5	11·0	14·0	19·0
2·1	2·7	14·0	17·0	2·5	3·0
1·0	1·3	1·5	2·0	6·4	8·0
1·2	1·5	2·4	3·0	1·8	2·3
6·0	8·0	0·5	0·6	2·4	3·0
1·2	1·5	3·5	4·5	0·7	0·9
1·6	2·0	4·0	4·8	2·8	3·5

The writer concludes therefore that Hunter's and White's modifications are no improvement and suggests the universal adoption of Thannhauser and Andersen's technique as outlined by McNee and Keefer, unless and until further work results in the introduction of an undeniably more accurate method.

As pointed out by Harrison [1930] however the Thannhauser and Andersen technique is unsatisfactory if there is gross haemolysis, because the haemoglobin is not completely precipitated by the addition of alcohol and ammonium sulphate. In that case it is best to repeat the test with fresh non-haemolysed serum; alternatively the original Van den Bergh's method may be used, in which the 2 volumes of added alcohol do precipitate the haemoglobin, but, as has been shown, such results may be seriously low.

SUMMARY.

Out of the several procedures suggested for Van den Bergh's estimation of bilirubin in blood the method described by Thannhauser and Andersen and the concentration of cobalt sulphate (2·161 %) used by McNee and Keefer are selected as the most suitable for clinical purposes.

I am indebted to Dr G. A. Harrison for his suggestions, his help in compiling this paper and for the facilities provided in his laboratory. The work was done with the aid of the Prof. Stokvis' funds, University of Amsterdam, and with a grant from the Cassel Foundation.

REFERENCES.

Harrison (1930). Chemical methods in clinical medicine, p. 247. (London.)
Hunter (1930). *Brit. J. Exp. Path.* **11**, 407.
McNee and Keefer (1925). *Brit. Med. J.* ii, 52.
Thannhauser and Andersen (1921). *Deutsch. Arch. klin. Med.* **137**, 179.
White (1932). *Brit. J. Exp. Path.* **13**, 76.

CLXIII. AN INVESTIGATION OF PROTEIN-FREE URINES GIVING A BIURET-LIKE REACTION.

BY EMANUEL GERARD GODFRIED.

From the Laboratory of Chemical Pathology, St Bartholomew's Hospital, London.

(*Received April 27th, 1935.*)

OCCASIONALLY when urine is added to Benedict's qualitative or to Fehling's solution the treated copper reagent becomes a deeper blue at room temperature. If the urine is superimposed carefully a reddish-purple ring is observed; it is this reaction, and not the violet colour due to a biuret reaction with urine containing protein, that has been studied.

When the fresh urines of healthy and diseased persons are examined carefully by this ring test, it is found that a large minority of both groups give positive reactions of varying intensity. It is important that the urine shall be relatively fresh, for the reaction diminishes in intensity in a few hours and in most cases becomes negative overnight.

This, though not generally known, is an old observation; Ralfe [1883] stated that the urine should be recently passed, for if kept overnight the reaction became negative, a fact which he suggested might be due to putrefactive changes. Ralfe and the older workers of course used Fehling's reagent which tends to give a slightly less marked test than Benedict's solution, but otherwise it is immaterial which of the two is employed; both copper reagents have been used in the present work, but Benedict's much the more frequently. This being understood, for convenience in subsequent description the test will be referred to as the "cold Benedict reaction".

For a long time peptone was believed to be the cause of the urinary "biuret test"; it will be shown that the substance cannot be peptone, and that, as Salkowski [1897] and Stokvis [1897] clearly recognised, the reaction should not be mistaken for a biuret test. The alternative suggestion of the above investigators, that urobilin was responsible, likewise cannot be supported.

Preliminary observations on the "cold Benedict reaction".

That a positive test cannot be due to peptone is shown by the following observations. When a positive urine is heated at 100° for 5 minutes and cooled, the reaction becomes negative. If now a few drops of tincture of iodine are added a reddish-purple ring does appear (see below under "Dybbuc test"). Saturating the urine with ammonium sulphate does not precipitate the substance responsible for the above tests.

If the biuret test is performed in the ordinary way (*e.g.* 3 parts urine, 1 part 40 % NaOH and 1 % $CuSO_4$ drop by drop) it is negative; only when a weaker alkali is employed is the reaction positive (see later). In other words the reaction is not a biuret test in the sense that this is employed for testing for proteins, proteoses or peptones.

That a positive test cannot be due to urobilin is shown by the following observations. Many urines giving positive cold Benedict reactions do not contain urobilin as judged from spectroscopical examination and Schlesinger's test, and conversely many urines which do contain urobilin give negative cold Benedict reactions. The reddish purple colour of the developed reaction is not extracted by amyl alcohol, benzene, carbon tetrachloride, chloroform, ether, ethyl acetate, toluene or xylene; spectroscopically the solution shows an ill-defined absorption of the green stretching from about its centre up to the E line and not a definite band in the position of that of the copper-urobilin compound. Many positively reacting urines give a negative result with Ehrlich's reagent (*p*-dimethylamino-benzaldehyde) for urobilinogen.

Positive cold Benedict reactions were obtained in some urines of both sexes, of both children and adults, in health and in a miscellaneous group of diseases. Positive results were not obtained in the urines of rabbits, guinea-pigs, rats or cats, though animals' urines have not been extensively studied.

No parallelism was established between the intensity of urinary tests for indican, or uroerythrin and the cold Benedict reaction. Solutions of pure creatine in water gave negative results.

The impression was gained that urine which has been stored in the urinary bladder for a short time only gives stronger reactions than urine which has been stored there for relatively long periods. One of the assistants in the laboratory usually passed urine giving a marked reaction; a specimen collected over a period of say 15 minutes gave a far stronger reaction than one collected over a period of say 2–3 hours; the effect of polyuria due to drinking and the possible influence of meals were carefully excluded.

Substitution of $CuSO_4$ and Na_2CO_3 solutions for the copper reagent.

The initial observations were made with Fehling's or Benedict's solutions; it was clearly desirable to simplify the reagents essential for the colour development. Neither copper sulphate solutions alone nor alkalis alone produced the colour on addition to the urine; both were necessary, and the colour development was better if the $CuSO_4$ were added before the alkali. After many experiments the following proportions appeared to be about optimum, *viz.* urine 5 ml., 1 % crystalline $CuSO_4$ 1 ml. and 5 % anhydrous Na_2CO_3 1 ml. On mixing these in the order given a rose or purplish red colour develops in a few seconds. If ammonia solution (sp. gr. 0·880) is employed as alkali, the colour is a deeper blue, but care has to be taken to avoid excess of ammonia. NaOH or KOH likewise can be substituted, but even greater care has to be taken to avoid excess, otherwise, as previously mentioned, the characteristic colour change does not occur at all.

Relation of "cold Benedict reaction" to "Dybbuc test".

Whilst working in Prof. Snapper's clinic in Amsterdam the following reaction was frequently performed by several of the pupils, including the writer, during routine chemical examination of urines. The cause of the reaction was not known, and the test was nicknamed "Dybbuc" (Hebrew for ghost or evil spirit). To about 5 ml. of urine 1 drop of ammonia solution (sp. gr. 0·880) is added, followed by tincture of iodine drop by drop until the colour is definitely reddish. One half treated with a solution of a zinc salt (*e.g.* 1 drop of 1 % $ZnSO_4$) becomes slightly more red. The other half treated with dilute $CuSO_4$ solution (*e.g.* 1 drop of 5 %) becomes reddish violet.

It naturally occurred to the writer that possibly the substance responsible for the cold Benedict reaction was the same as, or closely related to, the substance responsible for the "Dybbuc" test. Stokvis [1897] observed that the addition of iodine improved the colour obtained by treating urine with dilute solutions of KOH and $CuSO_4$.

In several hundred tests on the urines of more than 20 individuals a positive cold Benedict reaction has always been accompanied by a positive Dybbuc test. When urine which, initially positive, gives a negative cold Benedict reaction owing to keeping, heating at 100°, treatment with a small proportion of adsorbent charcoal or saturation with ammonium sulphate, it nevertheless still gives a positive "Dybbuc" test. On the other hand the cold Benedict reaction can again be made positive by adding a little iodine solution either before or after the addition of the urine to the Benedict's solution. This suggests that the substance responsible for the cold Benedict reaction may be a precursor of the substance responsible for the "Dybbuc" test.

In the hope of deciding whether the iodine merely causes oxidation other oxidising agents were substituted. Negative results were obtained employing in varying proportions solutions of each of the following: H_2O_2, $NaNO_2$, $KMnO_4$, $KClO_3$, $FeCl_3$ and Cl_2. Positive results could be obtained, but with less ease than with iodine, by using bromine water or potassium persulphate. It therefore looks as though it is not purely a question of simple oxidation.

The three tests, the cold Benedict reaction, treatment of the urine with $CuSO_4$ and Na_2CO_3, and the "Dybbuc" test were all used at the different stages in the subsequent attempt to decide the nature of the substance responsible for the colour development. The pigment is essentially red when extracted, the purple or violet tints in tests on urine being due to excess of $CuSO_4$. Further it is believed that the red pigments of the three tests are the same or closely allied; certainly in each case the same ill-defined absorption of the half of the green nearest the blue end of the spectrum is observed, and the red colour is unstable, fading slowly on standing, immediately upon acidifying or heating. The red pigment can be separated and concentrated by saturating the urine with ammonium sulphate and extracting with ethyl alcohol (*e.g.* 1/5 volume).

Further observations on the substance, X, responsible for the above tests.

X is destroyed by strong acids, and by H_2S in a few minutes. Strong alkali destroys it slowly, especially on heating. The other properties observed for X closely resemble those reported in the literature for substances in the urochrome group and may be summarised as follows.

(1) X is not extracted from urine by amyl alcohol, benzene, chloroform, ether or light petroleum.

(2) When a positively reacting urine is concentrated *in vacuo* below 50° to a syrup, both urochrome and X are extracted by ethyl alcohol. X therefore belongs to the "Extraktivstoffe" of Berzelius [Dombrowski, 1907–08, 1, 2].

(3) X is not adsorbed on the precipitate formed by treating urine with lead acetate at an acid reaction. Urochrome behaves similarly [Weisz, 1911; 1920, 1, 2; 1922; 1923; 1930]. $(NH_4)_2SO_4$, Na_2CO_3 or NaCl should be used for subsequent removal of the lead before testing the filtrate for X. The main bulk of the filtrate is precipitated by NH_4OH or NaOH because both urochrome [Weisz, 1920, 1, 2; 1930] and X are adsorbed on the alkaline lead precipitate. Both may be separated from the precipitate by adding the latter to a saturated solution of $(NH_4)_2SO_4$, followed by alcohol, when they pass into the alcohol layer; the strong alkali however soon destroys X.

(4) Saturation of the urine with ammonium sulphate precipitates urobilin which is removed by filtration. On treating the filtrate with ethyl alcohol urochrome is extracted [Garrod, 1894] and likewise X. The alcoholic extract is washed with saturated ammonium sulphate and evaporated to dryness *in vacuo.*

On extracting the residue with ethyl alcohol both urochrome [Garrod, 1894] and X are removed.

(5) Addition of mercuric acetate to the urine gives a precipitate on which urochrome [Thudichum, 1864; Garrod, 1894] and X are adsorbed. Both may be removed from the mercury precipitate in the same way as from the $Pb(OH)_2$ precipitate (see above).

(6) The urine is concentrated to a small volume *in vacuo* and dialysed. Neither urochrome [Fischer and Zerweck, 1924] nor X passes the membrane.

(7) Saturation with $(NH_4)_2SO_4$ followed by extraction with butyl alcohol removes urochrome [Drabkin, 1930, 1, 2] and X.

(8) Amyl alcohol extraction of acid urine removes substances of the urobilin group, but neither urochrome [Weisz, 1930] nor X.

(9) Butyl alcohol extracts urochrome [Drabkin, 1930, 1, 2] and X from acidified fresh urine. Drabkin washed his butyl alcoholic extracts and concentrated them *in vacuo*. In the writer's opinion this changes the nature of the urochrome for the following reasons: on standing the colour of the concentrated extract deepens considerably as Drabkin also noted; the final pigment appears to be no longer soluble in water but may be made so by simple washing with ether, which however must be carefully done for, as noted also by Drabkin, too long a contact with ether results in changes in the solubility of the pigment. The writer is doubtful whether unchanged urochrome is soluble in anhydrous butyl alcohol, though "urochrome" prepared by Drabkin's method is.

Butyl alcoholic extracts of X similarly washed and concentrated *in vacuo* yield material which no longer gives the tests for X.

(10) Alcoholic solutions of urochrome, prepared as described in either (2) or (4), yield a precipitate of pigment on addition of ether, but this "urochrome" precipitate has changed chemically for it is either insoluble or only partially soluble in ethyl alcohol [Garrod, 1894]. Likewise alcoholic solutions of X, similarly prepared, after treatment with ether no longer give the tests for X; neither the precipitate, nor the filtrate nor the two combined give a positive reaction.

(11) Charcoal adsorbs urochrome from urine [Hohlweg, 1908; Salomonsen, 1908; Mancini, 1908; Klemperer, 1903; 1904] and also X. Both can be extracted from the charcoal by 10 % NH_4OH in 50 % ethyl alcohol; mere traces are extracted by ethyl alcohol or methyl alcohol alone. Alternate washings with large volumes of acetone and of water remove urochrome partially and X completely.

Attempts to isolate X.

After the above study of its properties, the following procedure was employed in attempts to isolate X. The quantities of reagents given below are for 1000 ml. portions of urine.

The acid urine was treated with 250 ml. of saturated normal lead acetate and filtered. Lead was removed from the filtrate by excess of saturated ammonium sulphate. The second filtrate was shaken with about 50 g. of charcoal for 5 minutes. The precipitate was collected on a Büchner funnel and washed with large volumes of distilled water (filtrate and washings contained no X) and was then washed alternately five times with about 100 ml. portions each of acetone and of water, about 1000 ml. in all. The aqueous acetone solution was refiltered and concentrated to a small volume (about 20 ml.) below 40° under reduced pressure. The concentrate was submitted to one of the two following procedures.

(*a*) When the concentrate was submitted to chromatographic analysis in a column of $CaCO_3$, adsorption of pigment occurred in one layer only, and this layer gave strong reactions for X. In a series of experiments the deeper the pigmentation the stronger were these reactions. $BaCO_3$ and kaolin give less satisfactory adsorption.

(*b*) The concentrate was taken to dryness in a vacuum desiccator over $CaCl_2$. The yellow-brown residue was repeatedly submitted to prolonged extraction with anhydrous butyl alcohol in which a portion slowly dissolved. The butyl alcoholic extract gave with water, NH_4OH and iodine a beautiful red colour, and the usual subsequent colour changes with zinc and copper salts. The insoluble residue on solution in water gave negative tests for X. All attempts to concentrate the butyl alcoholic extract, or to separate X from it, resulted in partial or complete destruction of X.

The above procedure has been carried out on more than ten occasions. It has also been tried on urines which initially gave negative tests for X, when the final butyl alcoholic extract, if faintly yellow gave definite but weak reactions for X, but if colourless gave negative reactions.

It looks therefore as though X is either a pigment of the urochrome group or a substance associated with urochromes throughout the process of separation.

Opinions differ as to whether urochrome is a chemical entity or embraces a group of unknown substances [Weisz, 1920, 1, 2]. In the literature a number of pigments have been described which are supposed to be identical with urochrome because they are not precipitated by saturation with ammonium sulphate, and after such treatment are extracted from the filtrate by ethyl alcohol. The author has prepared "urochrome" from faeces [Garrod, 1903], "lactochrome" from milk [Palmer and Coolidge, 1914] and "protochrome" from peptone [Pelkan, 1920]. All these preparations gave negative tests for X.

SUMMARY.

1. The biuret-like reaction in protein-free urines which for many years has been regarded as due either to peptones or to urobilin has been further studied.

2. In agreement with certain authorities it is recognised that the reaction is not a biuret test in the usual sense.

3. It is shown that the reaction is not due to peptones or urobilin.

4. It is concluded that the substance responsible for the reaction is either a pigment of the urochrome group, or is associated with urochromes throughout the processes of separation.

I am greatly indebted to Dr G. A. Harrison for suggesting this investigation in which he himself had made a few preliminary observations, for the facilities provided in his laboratory and for his help in writing this paper. The work was undertaken with the aid of the Prof. Stokvis' funds, University of Amsterdam, and with a grant from the Cassel Foundation.

REFERENCES.

Dombrowski (1907–08, 1). *Z. physiol. Chem.* **54**, 188.
—— (1907–08, 2). *Z. physiol. Chem.* **54**, 390.
Drabkin (1930, 1). *J. Biol. Chem.* **88**, 433.
—— (1930, 2). *J. Biol. Chem.* **88**, 443.
Fischer and Zerweck (1924). *Z. physiol. Chem.* **137**, 176.
Garrod (1894). *Proc. Roy. Soc. Lond.* **55**, 394.
—— (1903). *J. Physiol.* **29**, 335.
Hohlweg (1908). *Biochem. Z.* **13**, 199.
Klemperer (1903). *Berl. klin. Woch.* **40**, 313.
—— (1904). *Verhandl. Berl. med. Ges.* **34**, 140.
Mancini (1908). *Biochem. Z.* **13**, 205.
Palmer and Coolidge (1914). *J. Biol. Chem.* **17**, 251.
Pelkan (1920). *J. Biol. Chem.* **43**, 237.
Ralfe (1883). *Brit. Med. J.* i, 905.
Salkowski (1897). *Berl. klin. Woch.* **34**, 353.
Salomonsen (1908). *Biochem. Z.* **13**, 205.
Stokvis (1897). *Z. Biol.* **34**, 466.
Thudichum (1864). *Brit. Med. J.* 509.
Weisz (1911). *Biochem. Z.* **30**, 333.
—— (1920, 1). *Biochem. Z.* **102**, 228.
—— (1920, 2). *Biochem. Z.* **112**, 61.
—— (1922). *Biochem. Z.* **133**, 331.
—— (1923). *Biochem. Z.* **134**, 269.
—— (1930). *Deutsch. Arch. klin. Med.* **166**, 331.

CLXIV. CONSTITUENTS OF HUMAN SEMINAL PLASMA.

By MAURICE WALTER GOLDBLATT.

From the Sherrington School of Physiology, St Thomas's Hospital, London.

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The chemical description of human seminal fluid is far from complete, and except for isolated observations few quantitative data were available until recently. Landois and Stirling [1891] describe it as being neutral or alkaline and containing, in addition to 82 % water and 2 % salts (phosphates, sulphates, carbonates and chlorides), serum albumin, alkali albuminate, nuclein, lecithin, cholesterol and phosphorised fat. Slowtzoff [1916], cited by Roger, gives 90·3 % water, 0·9 % ash, 8·8 % organic matter, 2·58 % protein and 0·21 % lipoids. The ash is stated to contain K, Na, Ca, Mg, P, Fe and S; Ca and P being most abundant. The protein is described as albumin, globulin, nucleoprotein, nuclein and albumose. Cholesterol, choline and spermine are also stated to be present. McCarthy *et al.* [1928] made an extensive series of analyses of fluid obtained by massage of the seminal vesicles and prostate. It is certain that the fluid thus obtained contains a much greater proportion of prostatic secretion than the fluid normally ejaculated. Some of the analytical figures given by these authors are so widely different from those obtained with seminal fluid that this statement seems justified.

In a recent publication Huggins and Johnson [1933] have presented the results of analyses of various fluids obtained from the male genital tract: spermatocele fluid, semen, semen after vasotomy and seminal vesicle fluid. As we are here interested only in the seminal fluid, we shall refer to the results of these workers only in so far as they affect our knowledge of this fluid. They found that the volume and composition of semen from vasotomised men were not very different from those of semen from normal men, thus indicating that the contribution of fluid from the testes and epididymis is not considerable. They also found that whereas semen and seminal vesicle fluid are very rich in glucose and inorganic phosphorus, prostatic fluid contained no glucose at all and very little inorganic phosphorus. The fluid from the seminal vesicles possessed a composition not greatly different from that of semen itself, so that the authors conclude that the main contribution to semen is from the seminal vesicles. The average values for the constituents of semen calculated from the figures given by these workers are: p_H 7·26, non-protein N 66·3 mg./100 ml., CO_2 14·6 mM, Cl 42·6 mM, glucose 295 mg./100 ml., inorganic P 65·6 mg./100 ml., calcium 20·8 mg./100 ml. There are certain difficulties in making some of these analyses to which we shall refer later. It is of interest to compare these findings with the statement made by Marshall [1922] that the amount of calcium in one ejaculation is 0·01 g., *i.e.* the amount of calcium in 50 ml. semen according to the results of Huggins and Johnson. Unless the seminal fluid contains forms of calcium which cannot be precipitated by ammonium oxalate, the figure given by Marshall is 10 times too high.

The question of the nature of the proteins in seminal fluid has presented certain difficulties because of the presence of non-coagulable protein.

It has been stated by several workers that choline is present in seminal fluid. Evidence against this statement is given in a paper on the pharmacological properties of seminal plasma by the writer [Goldblatt, 1935].

EXPERIMENTAL.

The specimens placed at our disposal were the results of normal coitus and were received in the laboratory at various times up to 12 hours after ejaculation. In all the specimens motile spermatozoa were very abundant and in no case was there evidence of bacterial decomposition.

Having regard to the variable content of cells and other microscopically visible material, it was decided to work only with seminal plasma, *i.e.* the fluid obtained after centrifuging the semen for 10 min. or so at about 6000 r.p.m. and decanting or pipetting the supernatant fluid. Plasma thus obtained is almost free from cells and suspended particles. Seminal plasma is stable for long periods and appears rather like a milky blood serum; it is opalescent and often of a pale yellow colour; it is not very viscid and it gives no deposit on standing; sometimes it is very turbid; it possesses the peculiar odour of semen, which becomes more intense on warming.

Reaction. The p_H of seminal plasma, determined colorimetrically, varied in different specimens from 7·5 to 7·8, but this range is certainly more alkaline than at the time of ejaculation. Huggins and Johnson [1933] used semen immediately after emission and at once covered with oil and found the p_H to be 7·26, great care having been taken to prevent contamination with urinary residues. Such a low p_H compared with blood is in accord with the high amino-acid, phosphate and lactic acid contents.

It is generally stated that in physiological conditions the alkalinity of the seminal fluid is necessary to prevent immobilisation of the sperm cells by the acid secretion of the vagina. It must be remembered that the vagina itself is free from glands and that it is supplied with mucus from the glands of the cervix. The production of acid in the vagina is due to the action of organisms on the glycogen of the vaginal epithelium. It seems probable also that the spermatozoa on entering the vagina produce a considerable amount of lactic acid between the period of ejaculation and complete loss of motility. The necessity for a powerful buffering mechanism in semen is therefore obvious and even a rough titration with $N/10$ HCl shows how efficient its buffering capacity really is. Being, compared with blood, relatively poor in protein it owes its high buffering capacity to bicarbonate and to an enormous inorganic phosphate content. It seems that from the point of view of p_H regulation the seminal fluid stands intermediate between blood and urine, the mechanisms of both being utilised.

Bicarbonate. If seminal plasma be treated with a little mineral acid carbon dioxide is liberated. Experiments with the Van Slyke constant pressure apparatus show that seminal plasma loses half its bound CO_2 on exposure to a vacuum, from which we may conclude that the bound CO_2 is all present as HCO_3^-, as was to be expected. The average value obtained for our series of specimens was 22 millimols CO_2 per litre; this value is rather higher than that given by Huggins and Johnson, *viz.* 14·6.

Chloride. Chloride was estimated by Van Slyke's method for blood. The values obtained with three specimens were 0·209, 0·187 and 0·193 g./100 ml., expressed as NaCl, thus giving an average of 0·197 g./100 ml. The closeness of these results suggests that the salt content of seminal plasma may be normally maintained between as narrow limits as is the blood plasma chloride.

For prostato-vesicular secretion McCarthy *et al.* [1928] find 0·231 g./100 ml. for the chloride as NaCl whilst Huggins and Johnson [1933] give 0·164 for seminal vesicle fluid, 0·164 for semen after vasotomy and 0·355 for prostatic fluid. If we apply the figures of the latter workers to those of McCarthy *et al.* we find that the prostato-vesicular fluid obtained by the latter consisted of two parts vesicle fluid and one part prostatic fluid. The figures of Huggins and Johnson lead to the conclusion that, on the average, semen after vasotomy did not contain any prostatic fluid at all. An interesting possibility is thus presented that vasotomy may lead to an inhibition of the release of prostatic secretion. This is also suggested by the average figures of these authors for glucose and inorganic phosphorus.

Phosphate. Seminal plasma is very rich in phosphorus compounds. Attempts to use the technique of Briggs for the estimation of inorganic phosphorus led to unexpected difficulties; these were soon traced to the fact that the trichloroacetic acid filtrates were not protein-free. The same difficulty in obtaining protein-free filtrates is met with in each of the following methods of deproteinisation: Folin's tungstic acid, acidified sodium sulphate and colloidal ferric hydroxide, sodium hydroxide and zinc sulphate, 80 % alcohol. The soluble protein is proteose. In addition to this difficulty there is also the matter of the presence of spermine phosphate which is readily soluble in trichloroacetic acid. We shall consider the latter question first.

Behaviour of spermine phosphate towards Briggs's reagents.

In the first experiments we used spermine phosphate (kindly supplied by Messrs Hofmann La Roche) dissolved in dilute alkali. On adding the reagents in the proportions used for blood plasma a yellow crystalline precipitate was formed and a blue colour gradually developed in the turbid mixture. Removal of the precipitate and comparison of the blue colour with standards of KH_2PO_4 showed that from 1·35 and 0·68 mg. spermine phosphate (*i.e.* 0·16 mg. and 0·08 mg. P) only 0·07 and 0·04 mg. "inorganic" P was estimated. It was quite clear that the Briggs technique precipitated a great deal of the spermine as a molybdate insoluble in the conditions of the estimation.

Now the structure of spermine phosphate leads us to expect it to behave like an inorganic phosphate, so the following experiments were carried out to determine the proper conditions, using quantities of spermine of the same order as that found in seminal plasma. For these experiments we used a specimen of spermine diphosphate kindly supplied by Dr G. A. Harrison of St Bartholomew's Hospital and prepared by him from human tissues [1931].

A solution of this salt in 4 % trichloroacetic acid was prepared containing 1 mg. per ml. The formula for spermine diphosphate is $C_{10}H_{26}N_4 . 2H_3PO_4, 6H_2O$, *i.e.* it contains 21·3 % water of crystallisation and 12·25 % total P. Hence 1 mg. spermine diphosphate should yield 0·123 mg. "inorganic" P if it behaves as an inorganic phosphate.

(*a*) 2 ml. of the solution were wet-ashed and the total phosphorus determined by Briggs's method was found to be 0·256 mg. (theoretical 0·245).

(*b*) To determine the proportions of acid molybdate which, for quantities of spermine phosphate likely to be met with in human seminal plasma, would not produce either a turbidity or a precipitate, the following mixtures were prepared.

Spermine phosphate solution ml.	4 % trichloro-acetic acid ml.	Briggs's acid molybdate ml.	Water ml.	Remarks
2·0	—	2·0	3·0	Precipitate
2·0	3·0	2·0	—	Precipitate
2·0	—	2·0	—	Turbidity
0·5	1·5	2·0	—	Turbidity
0·25	1·75	2·0	—	Turbidity
1·0	—	2·0	—	No turbidity
2·0	—	4·0	—	No turbidity

It is clear, therefore, that the use of Briggs's molybdate in trichloroacetic acid filtrates of human seminal plasma containing spermine will produce precipitates if the 2 : 7 proportion of molybdate be used as it is for blood plasma filtrates. If, however, two parts of molybdate be added to one part of the spermine-containing filtrate no precipitate or turbidity due to the spermine will be produced.

On mixing 1 ml. of the above spermine diphosphate solution with 2 ml. molybdate solution and then adding 1 ml. of the quinol (0·5 %) and 1 ml. of the sodium sulphite (20 %), a pure clear blue gradually developed which corresponded to an inorganic phosphorus content of 0·125 mg. (theoretical 0·123 mg.). Similarly 2 ml. of the solution of spermine diphosphate and 4 ml. of the molybdate followed by 1 ml. of the quinol and sulphite gave a blue corresponding to 0·227 mg. inorganic phosphorus (theoretical 0·245 mg.). The development of the colour ran exactly parallel with that of the standard KH_2PO_4, so that it is justifiable to suppose that spermine phosphate acts towards the Briggs reagents as an inorganic phosphate. If we take 3 mg. per ml. as the upper limit of the spermine phosphate content of seminal plasma, the above proportions of molybdate should obviate any difficulties from the formation of insoluble spermine phosphomolybdate, provided we take volumes of trichloroacetic acid filtrates equivalent to 2/3 ml. of seminal plasma. But when dealing with seminal plasma complications still arise from the presence of protein in the trichloroacetic acid filtrate. Huggins and Johnson [1933] refer shortly to a turbidity on adding acid molybdate to the trichloroacetic acid filtrate and state that they removed it by ultrafiltration through a collodion sac under a negative pressure of 150 mm. Hg. We must therefore consider what effect the presence of soluble protein (proteose) has upon the estimation of inorganic phosphorus.

Effect of proteose on the estimation of inorganic phosphorus.

A series of mixtures of 1 % Witte's peptone (filtered and shown to contain proteose and peptone) and KH_2PO_4 were prepared so that 2 ml. contained 0·1 mg. P and 0, 2, 4, 6, 8 and 10 mg. of the protein. To 2 ml. of these mixtures were added 10 ml. 20 % trichloroacetic acid. The protein precipitates were filtered and refiltered until the filtrate was as clear as possible. To 5 ml. of the filtrates were added 2 ml. acid molybdate, 1 ml. of quinol and 1 ml. of sulphite. In all except the first tube turbidities of increasing intensity appeared according to the amount of protein in the original tubes. After about 30 mins. the contents of each tube were filtered and the clear blue filtrates compared with the blue of the first filtrate. The following figures give the amounts of inorganic phosphate in the filtrates: 0·042 (no protein), 0·040, 0·035, 0·035, 0·030, 0·028 mg. It is clear that the losses were due to the formation of insoluble phosphomolybdate of protein. If acid molybdate be added to a solution of Witte's peptone a precipitate is formed which readily dissolves in excess of the reagent; but if a trace of phosphorus be added to the protein solution before addition of the molybdate there is formed a precipitate which does not dissolve in excess of the reagent. In the 5 ml. portions of the trichloroacetic acid filtrates used in the above experiment there were increasing amounts of dissolved protein and so we expect increasing amounts of the phosphate to be lost. If the losses in inorganic phosphorus be plotted against the original concentration of protein the points fall approximately on a straight line. It is clear, therefore, that unless we know the amount of proteose in the filtrates obtained with seminal plasma an undetermined amount of inorganic phosphate will be lost in removing the precipitate formed on adding the acid molybdate. We have no data on the amount of proteose in seminal fluid but we suspect it to be present in very considerable amount. There is no doubt that the values obtained by other workers and ourselves for inorganic phosphorus in seminal fluid are considerably lower than the actual content. We proceed as follows: 10 ml. 20 % trichloroacetic acid are added to 2 ml. seminal plasma and after vigorous shaking allowed to stand for an hour. The mixture is now filtered and refiltered until a clear filtrate is obtained. This filtrate invariably gives a positive biuret reaction. To 2 ml. of the filtrate are added 4 ml. of the acid molybdate; a heavy yellowish turbidity forms at once and gradually settles out as an amorphous precipitate. Repeated filtration will usually yield a protein-free filtrate but sometimes we find it necessary to add a little $CaCl_2$ (anhydrous) and re-filter. Addition of the Briggs reagent to an aliquot part of the filtrate now gives a clear blue which can be compared with standards in the usual way.

In this way we obtain values for "inorganic" phosphorus which include all the spermine phosphate, but, as we have pointed out, these values are too low. The averages of our series of specimens were:

Total acid-soluble phosphorus	95 mg./100 ml.
"Inorganic phosphorus"	70 mg./100 ml.

If we take Harrison's figures for the spermine phosphate content of human semen, *viz.* 112–268 mg./100 ml., then it is not excessive to attribute some 15 to 30 mg./100 ml. P of the "inorganic" phosphorus to this substance. This leaves 40–50 mg./100 ml. as the true inorganic phosphate subject to the reservations considered above.

Huggins and Johnson [1933] found that prostatic fluid contained only 3·4 mg./100 ml. inorganic phosphorus. Harrison [1933] has shown that the prostate is richer in spermine than any other organ in the body; the average figure given was 583 mg. spermine phosphate per 100 g. wet weight. It is not unlikely that most of the phosphate in prostatic secretion is in the form of spermine phosphate. Now we have seen above that some 15–30 mg. of the "inorganic" phosphate of seminal fluid are due to spermine phosphate and there is no doubt that the prostatic contribution to seminal fluid is small in proportion to that of the seminal vesicles; hence we must conclude that the vesicles contain (secrete) spermine phosphate. The facts that the testes and epididymis contribute very little fluid to seminal fluid, that the testes are relatively poor in spermine (only 60 mg. per 100 g. wet weight according to Harrison [1933]) and that spermatocele fluid is free from spermine lead us to the conclusion that the major part of the spermine in seminal fluid arises from the seminal vesicles. Fluid from the distended seminal vesicles of young men was obtained by Huggins and Johnson [1933] and found to contain 45 mg./100 ml. inorganic phosphorus, but they did not consider the spermine content.

There is no direct evidence as to the function of all this phosphorus but it seems reasonable to consider the great change in chemical environment experienced by the sperm on ejaculation as related to the necessity for active movement in the female genital tract and that the phosphates ensure a very efficient buffering action. As to the function of spermine phosphate nothing is known.

Calcium. The only other inorganic constituent of seminal plasma we examined was calcium. Using the ordinary technique of Kramer and Tisdall for serum calcium we found an average value of 24·5 mg./100 ml. This agrees well with the finding by Huggins and Johnson of 20·8 mg./100 ml. The origin of this very high concentration of calcium is most probably the prostate. This is supported by the finding of McCarthy *et al.* [1928] of 66 mg./100 ml. calcium in prostato-vesicular secretion. The former authors, however, give only 23·3 mg./100 ml. as the calcium content of seminal fluid after vasotomy, a result due probably to the small fluid contribution made by the prostate.

The forms in which the calcium exists in seminal fluid are probably calcium hydrogen phosphate, calcium bicarbonate and a combination with protein. The low phosphate content of prostatic secretion indicates that very little calcium can come from this source as the hydrogen phosphate but we have no certain data as to the partition of calcium among the three possible combinations.

Urea. It is not surprising to find urea in seminal fluid having regard to its ready diffusibility into all tissues but its function here, if any, is not easily understood. The great concentration of other blood constituents in the seminal plasma may involve urea more or less accidentally. Be that as it may, the concentration of urea in this fluid is roughly twice that in blood. The average value in our series was 72 mg./100 ml., estimated by hydrolysis with soya bean and aeration into standard acid. Of course very small contaminations with urine would give false values but the evidence is against such contamination since similar values are obtained even when the urethra was washed with dilute boric acid before ejaculation [McCarthy *et al.*, 1928].

Reducing substances. In estimating the reducing power of seminal plasma the difficulty of deproteinising, already referred to, at once introduces complications. We have used MacLean's method (a Cu method) and that of Hagedorn and Jensen (ferricyanide method). The latter method gives values which are certainly unreliable owing to the much greater proportion of protein which escapes precipitation. The former method gives filtrates which contain only a faint trace of protein and, provided a "blank" determination be made in the cold, reliable values are obtainable. The following values were obtained with the seminal plasma of normal and diabetic subjects.

Normal, 206, 277, 226, 211, 282, 380, 291 mg./100 ml. as glucose.
Diabetic, 628, 569, 576, 669, 681 mg./100 ml. as glucose.

Thus normal seminal plasma contains between two and three times the amount of reducing substances found in normal blood. The values for the diabetic subjects are probably more or less misleading, since traces of glucose are always to be found in the urethra of the glycosuric. There can be no reasonable doubt that the sugar in the seminal fluid must be regarded as a nutrient for the spermatozoa and it is easy to demonstrate the utilisation of the sugar if the sperm cells are kept alive. If the sperm cells are killed by heat, dilution or by the addition of a drop of toluene no fall occurs in the reducing power of the fluid. Nor does the reducing power change in the seminal plasma if it is kept sterile. In the following experiment a fresh specimen of human seminal fluid was obtained in a sterile tube, divided into three parts: one part was heated to kill the sperm, another part incubated in a water-bath at 38° and the remaining part centrifuged and the supernatant plasma used. Estimations of reducing substances were made in each part at once and after 20 hours' incubation.

	Heated fluid	Plasma	Unheated fluid
At once	291	280	291 mg./100 ml. as glucose
After 20 hours' incubation	289	280	72

McCarthy *et al.* [1928] also recorded glycolytic activity in incubated seminal fluid and found that there was a large increase in lactic acid but that this increase did not account for the loss in sugar. This production of lactic acid is probably an important factor in the poor survival of spermatozoa in natural seminal fluid *in vitro* however carefully sterility, temperature and oxygen supply are controlled. Failure of the buffering capacity comes about sooner or later but generally within 12 hours.

The source of the sugar in the seminal fluid seems to be the seminal vesicles, for the prostatic fluid has no reducing power at all.

Lactic acid. Seminal fluid contains lactic acid in large amounts. The acid is present in the freshly ejaculated fluid and increases on incubation. We used the method of Friedemann, Cotonio and Shaffer for the determination of this substance in the tungstic acid filtrate after treatment with copper sulphate and calcium hydroxide. The following values were obtained with different specimens of fresh seminal fluid, the spermatozoa having been killed soon after ejaculation:

92, 80, 104, 87, 103 mg./100 ml.

The increase in lactic acid during incubation of seminal fluid containing living sperm cells is shown in the following:

	Reducing power as glucose mg./100 ml.	Lactic acid mg./100 ml.
At beginning	291	80
After 20 hours' incubation at 38°	72	140

There is clearly a great discrepancy between the lactic acid production and the lost glucose. How the lactic acid is produced in the vesicles it is hard to say. The few spermatozoa which may be present in this situation do not appear to be sufficient to account for so high a lactic acid value nor can we attribute it to an enzymic action on the glucose, for the seminal plasma, freed from spermatozoa, does not show any increased lactic acid content after incubation. Nor can we suppose that it is formed in the prostate (because prostatic secretion is free from reducing substances) unless we consider that it may arise from a non-carbohydrate source (*e.g.* amino-acid). If it derives directly from the blood we must attribute remarkable powers of selective concentration to the seminal vesicles. Teleologically it appears useless to produce large quantities of lactic acid in a medium where its presence later will be inimical to the survival of the sperm cells. There are, of course, considerable numbers of leucocytes and other cells in seminal fluid and these, as in blood, may be partly responsible for the glucose-lactic acid reaction. The matter is not easy to investigate and is in a state of obscurity.

Cholesterol. This substance can be readily estimated in seminal plasma by the usual methods for serum cholesterol. Sometimes other substances are extracted by the chloroform and may interfere with the purity of the colour obtained with the acetic anhydride. The average value for our series was 82 mg./100 ml.

In the following table we give a comparison of the composition of blood plasma and seminal plasma.

Composition of blood and seminal plasma.

	Blood plasma 100 ml.	Seminal plasma 100 ml.
p_H	7·4	7·2–7·3
NaCl (g.)	0·59–0·60	0·20
CO_2 as ml./100 ml.	50– 70	50
Inorganic P (mg.)	3– 4	40– 50
Acid-soluble P (mg.)	3– 4	95
Spermine P (mg.)	—	15– 30
Calcium (mg.)	10	24– 25
Glucose (mg.)	100	200–300
Urea (mg.)	30	72
Lactic acid (mg.)	15– 20	90–100
Cholesterol (mg.)	150–200	80

Proteins. Seminal plasma gives all the protein colour reactions. When slightly acidified with 1 % acetic acid it gives a slight precipitate; this probably indicates the presence of a small amount of nucleoprotein. It must be remembered that the mucinous matter secreted by the accessory glands is removed in a stringy mass when the semen is centrifuged. On dilution of seminal plasma with a large excess of water a slight precipitate of globulin is formed. On filtering off the globulin a considerable amount of coagulable protein is readily demonstrable in the filtrate; this we take to be albumin.

The reaction at which maximum coagulation is obtained is about p_H 5·5 but the amount of coagulable protein seems to vary a great deal in different specimens. Removal of the coagulable protein always yields a richly proteinous filtrate. This non-coagulable protein is referred to in the older literature as propeptone and sometimes as hemialbumose. Marshall [1922] refers to an albumose-like substance and remarks that "the statement that this protein is an albumose is probably not correct, since albumoses have never been found to occur in the living cell". Posner [1892] refers to the non-coagulable protein of human seminal fluid as propeptone and finds that it is absent from spermatocele fluid which contains enormous numbers of fully developed but non-motile spermatozoa; he concludes that the propeptone is elaborated in the accessory glands and not in the testes. Vertun [1899] also analysed spermatocele fluid; in his case actively motile spermatozoa were present; the fluid contained albumin and a trace of globulin but a variety of tests showed that it was free from albumose, from which he concluded that the presence of albumose was of no importance in the maintenance of the motility of the spermatozoa.

Our own findings with every specimen of seminal plasma support the view that there is proteose present. On removing the coagulable protein by boiling at p_H 5·5 and filtering, the filtrate gives a pink biuret reaction; half-saturation of the filtrate with ammonium sulphate gives a considerable precipitate the filtrate from which gives another precipitate on full saturation with ammonium sulphate. The filtrate after full saturation gives no protein reactions. We were able partly to separate the primary and secondary proteoses by ultrafiltration. Human seminal plasma was ultrafiltered through a collodion membrane under a pressure of 50 mm. Hg. A yellowish clear filtrate was obtained which was alkaline to litmus, contained no coagulable protein, gave a pink biuret reaction, a positive salicylsulphonic acid reaction, a precipitate with nitric acid which disappeared on warming and reappeared on cooling but no precipitate with copper sulphate. The ultrafiltrate on half-saturation with ammonium sulphate gave a copious precipitate the filtrate from which still contained protein; all the protein was removed on full saturation of the filtrate. The ultrafiltrate, therefore, contained both primary and secondary proteoses. The fluid left in the collodion sac was acidified and boiled to remove the coagulable proteins; the filtrate from this was rich in protein but gave no precipitate on half-saturation with ammonium sulphate but a copious one on full saturation. It seems, therefore, that the primary proteose had all passed through the collodion filter but the secondary proteose had done so only in part. Some specimens of seminal fluid contain no primary proteose at all but there is never any doubt about the secondary proteose. At least two secondary proteoses are present which can be demonstrated as follows. Remove the coagulable proteins by boiling at p_H 5·5; filter and half-saturate the filtrate with ammonium sulphate; filter and add a half-volume of saturated ammonium sulphate; a copious precipitate is produced. This precipitate can readily be filtered off, dissolved in water and precipitated with two volumes of absolute alcohol (the alcohol precipitates the ammonium sulphate as well as the protein but a pink biuret reaction is readily obtained if excess of alkali is used). These facts show the presence of thioalbumose or a substance closely resembling it. On removing the thioalbumose we obtain a water-clear filtrate which gives a slight turbidity or precipitate on full saturation with ammonium sulphate, removal of which yields a non-protein filtrate. The protein nature of the last precipitate is readily established by dissolving in water and obtaining a pink biuret reaction. We did not further identify this substance but it seems likely that it is synalbumose.

We conclude that the following proteins are present in seminal fluid: mucin, nucleoprotein, albumin, globulin (traces), proteoses but no peptone. The proteoses may be both primary and secondary but in some specimens primary proteose may be absent. Secondary proteoses are always present and consist of thioalbumose and synalbumose or bodies closely resembling them.

It is now clear why the usual methods of protein precipitation used in blood analysis do not yield non-protein filtrates when applied to seminal plasma. These methods if applied to a solution of Witte's peptone, containing proteose and peptone, always yield filtrates giving the reactions of proteose. The same remarks apply if the precipitation is carried out in 80 % alcohol.

Alcohol-soluble material in seminal plasma.

One volume of seminal plasma is mixed with four volumes of absolute alcohol and well shaken. The copious white precipitate is removed by filtration and the alcohol removed from the filtrate by distillation at 42° under reduced pressure. The whitish residue when taken up in water yields a turbid suspension and gives the following reactions: it is alkaline (sometimes neutral), biuret—pink, Millon—weakly positive, xanthoproteic—no precipitate but turns yellow on heating and orange on adding ammonia, Molisch and glyoxylic—negative, ammonia does not give a precipitate, the Pauly, Sakaguchi and ninhydrin reactions are all strongly positive. The intensities of the last three reactions are so out of proportion to that of the biuret reaction that it seems possible that there are free iminazole and guanidine compounds present. This possibility has not as yet been followed up but it is suggestive that all these reactions become negative in the extract after a relatively short period of dialysis and that after precipitation of all the proteins of seminal plasma by full saturation with ammonium sulphate the filtrate still gives these reactions. We have already referred to the results of other workers that seminal fluid is very rich in amino-acids and this may explain our findings. We conclude, therefore, that the alcohol extract contains proteose and probably a considerable amount of amino-acids.

The presence of proteose in seminal plasma suggests that a proteolytic enzyme may be present or that the spermatozoa may have the power of splitting proteins. The latter seems improbable in view of the absence of proteose from spermatocele fluid containing motile sperm. The absence of peptone argues against any ordinary peptic activity but the matter requires further investigation. Tested upon calcified milk seminal plasma gives no evidence of the presence of a proteolytic ferment.

Diastase. Karassik [1927] reports the presence of diastase in human prostatic fluid and McCarthy *et al.* [1928] found it in prostato-vesicular fluid. The wide distribution of this enzyme renders its presence in seminal plasma not unexpected. We studied the diastatic power of fresh seminal plasma in buffered solution as follows:

Tube no.	1	2	3
0·1 % starch (ml.)	5	5	5
1·0 % NaCl (ml.)	2	2	2
KH_2PO_4-NaOH buffer (ml.)	2	2	2
p_H	6·6	5·8	8·0
Seminal plasma (ml.)	0·4	0·4	0·4
Time for complete digestion at 38° (min.)	45	65	No change in 2 hours

It appears, therefore, that a diastase is present which is active at reactions between p_H 6 and 7. A simple calculation shows that the diastatic activity of 3 or 4 ml. of seminal plasma is sufficient to produce an amount of simpler sugars from polysaccharide (*e.g.* glycogen from the vagina and cervix) more than sufficient to supply the needs of the spermatozoa for a considerable time.

Glycolytic activity. Seminal plasma has no glycolytic activity if all the sperm cells have been removed.

Thrombokinase. Coagulating agents have been stated to be present in the seminal secretions of rats, mice and guinea-pigs by Walker [1910] and others. A special gland exists in these animals which secretes a substance which coagulates the contents of the seminal vesicles and so brings about the vaginal plug formation well known in these animals. Walker could not find these glands in rabbits or bullocks. In human seminal fluid particles or aggregations are seen which suggest some sort of coagulation to this author but he does not state what is coagulated. Any close analogy to the fibrinogen-fibrin change in blood seems improbable but, although the amount of globulin in seminal plasma is small, we cannot say with certainty that something akin to fibrinogen is not present. One factor in the mechanism of blood coagulation can readily be demonstrated in seminal plasma, *viz.* thrombokinase. This was done as follows: to 4·5 ml. seminal plasma were added 27 ml. absolute alcohol; the mixture was well shaken and allowed to stand for an hour or so; the filtrate from this was freed from alcohol by distillation at 42° under reduced pressure and the residue taken up into 10 ml. water. A solution of prothrombase (Mellanby) containing 0·5 mg./ml. was prepared. The presence of thrombokinase was tested by the power to activate prothrombase to thrombase in the presence of $CaCl_2$; the thrombase activity was tested by the power to coagulate oxalated ox plasma. The prothrombase was first shown to be inactive. A mixture of 2 ml. prothrombase solution, 1 drop $N/5$ $CaCl_2$ and 1 drop of seminal plasma extract was placed in a water-bath at 38° for 2 mins. 0·1 ml. of the prothrombase solution thus treated added to 1 ml. oxalated plasma at 38° produced complete coagulation in 15 secs. An exactly similar experiment with a freshly prepared aqueous extract of bull's testis gave a coagulation time of 12 secs. An acetone extract of seminal plasma was also used in a similar experiment and gave a coagulation time of 45 secs.

It is thus clear that a thrombokinase of considerable activity is present in seminal plasma. The origin of this kinase may be the testis but we think its more likely origin is the broken down cells of several kinds which are always found in the seminal fluid. As to the function of kinase in seminal fluid we make no suggestion.

Miscellaneous observations.

(*a*) It seemed possible that seminal fluid might contain hormones having an influence on the female genital tract. Injection of 1 ml. fresh seminal fluid intravenously into young virgin rabbits did not, however, produce any changes in the uterus or ovaries during periods up to 48 hours after the injections. The animals showed no toxic effects but invariably the injections were followed by an almost immediate defaecation; for the effect on intestinal muscle see Goldblatt [1935].

(*b*) Successive intravenous injections of seminal fluid or alcoholic extracts thereof into rabbits had no effect on blood sugar.

(*c*) Seminal fluid does not give the nitroprusside reaction for glutathione or related bodies.

SUMMARY.

1. Human seminal plasma has been quantitatively analysed for certain constituents. The ratios of the concentrations of these in seminal plasma to their concentrations in blood plasma are as follows: chloride and cholesterol, 0·3–0·5; bicarbonate, 0·7–1·0; calcium, glucose, urea, 2·0–3·0; lactic acid, 5·0–6·0; acid-soluble phosphorus (inorganic, 50 %, spermine, 30 %, undetermined 20 %), 30.

2. The proteins of seminal plasma include: mucin (traces), nucleoprotein, albumin, globulin (traces), proteoses. The proteoses may be both primary and secondary; secondary proteose is always present. The secondary proteose, it is suggested, consists of thioalbumose and synalbumose or bodies closely resembling them. Peptone is never present in seminal plasma. The presence of proteoses interferes with several estimations.

Protamines and histones have not been found in seminal plasma.

3. Glycolysis can be demonstrated in seminal plasma only if active sperm cells are present. This is associated with a large increase in lactic acid but the latter is not sufficient to account for all the lost reducing power.

Proteolytic ferments have not been found in seminal plasma and hence the presence of proteose cannot be explained on enzymic grounds.

Diastase and thrombokinase have been demonstrated in seminal plasma.

4. Seminal plasma does not appear to contain hormones affecting either the genital tract or carbohydrate metabolism.

REFERENCES.

Goldblatt (1935). *J. Physiol.* **84**, 208.
Harrison (1931). *Biochem. J.* **25**, 1885.
—— (1933). *Biochem. J.* **27**, 1152.
Huggins and Johnson (1933). *Amer. J. Physiol.* **103**, 574.
Karassik (1927). *Z. ges. exp. Med.* **53**, 734.
Landois and Stirling (1891). Text-book of physiology. (C. Griffin and Co., London.)
Marshall (1922). The physiology of reproduction. (Longmans, Green and Co., London.)
McCarthy, Stepita, Johnston and Killian (1928). *J. Urology,* **19**, 43.
Posner (1892). *Zentr. mikr. Wiss.* **13**, 225.
Slowtzoff (1916). Cited by G. H. Roger, Traité de physiologie. (Masson et Cie, Paris.)
Vertun (1899). *Zentr. mikr. Wiss.* **31**, 529.
Walker (1910). *Bull. Johns Hopkins Hosp.* **21**, 182.

CLXV. THE FATTY ACIDS OF PIG LIVER.

III. A GENERAL ANALYSIS.

By EILEEN IRVING and JAMES ANDREW BUCHAN SMITH.

From the Department of Biochemistry, The University of Liverpool.

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In two earlier communications, Channon *et al.* [1934, 1, 2] described their investigations on the nature of the octadecenoic acids of pig liver and discussed in detail the bearing of their results on the question of the desaturation of fatty acids in that organ. In continuation of these experiments the original fatty acids which had been prepared from 100 kg. of pig liver were subjected to further analyses by different methods. They were treated on a scale sufficiently large to give the results something of that completeness which they require, if ever they are to contribute materially to the elucidation of problems concerning fat metabolism in the animal body.

In view of the recognised importance of the liver in fat metabolism, it is surprising to find how little serious work has been carried out so far to establish the precise nature of the fatty acids contained in the mammalian liver. The chief contributors to this subject have been Hartley [1909], who analysed the fatty acids from pig liver, discovering in them the highly unsaturated arachidonic acid, and Klenk and Schoenebeck [1932] who made a thorough examination of those present as glycerides and phosphatides in ox liver. Work in similar fields published from various laboratories in recent years, such as the discovery of a liquid saturated acid in cat's kidney by Turner [1931] and of other liquid saturated acids in tubercle bacilli by Anderson [1929] and Spielman [1934], as well as the evidence put forward by Bosworth and Brown [1933] and by Bosworth and Sisson [1934] for the presence in butter fat of arachidonic and lower unsaturated acids, indicates that a thorough investigation of the lipins from different parts of the animal body has already been too long delayed. There can be little doubt that results accumulating in the course of time from researches of this type will ultimately bring to light the essential details of certain biological processes of which for the present the explanation remains obscure. As a contribution to this end the present work was carried out.

EXPERIMENTAL.

The preparation of the fatty acids and their separation into saturated and unsaturated fractions by the Twitchell process, as modified by Hilditch and Priestman [1931] has already been described by Channon *et al.* [1934, 1]. The main points to be noted for the present communication are these. The original acids had an iodine value of 110 and a mean molecular weight of 299. By the lead soap-alcohol procedure the mixture was shown to contain 34 % of saturated acids (I.V. 2; mol. wt. 277), and 66 % of unsaturated acids (I.V. 153; mol. wt. 321).

Estimation of the saturated acids by the Bertram method.

This consisted of oxidising samples of both the original mixture and the unsaturated fraction with permanganate and precipitating the petroleum-soluble products twice as magnesium soaps [Bertram, 1927].

(*a*) *The original acids.* 5·035 g. gave 1·750 g. saturated acids, or 34·8 %.

(*b*) *The unsaturated acids.* 5·226 g. gave 0·197 g. saturated acids. This is equivalent to 3·8 % of the unsaturated fraction, or to 2·5 % of the original acids.

In summarising these results it can be said that the content of the saturated acids was found to be 34·8 % by the Bertram process alone, and 36·5 % (*i.e.* 34·0+2·5) by the two methods combined. That this higher figure of 36·5 % approximated more nearly to the truth was proved later by distillation methods.

Analysis of the saturated acids.

210 g. of methyl esters were prepared from the saturated acids and fractionally distilled at 2 mm. The fractions were saponified, traces of unsaponifiable matter removed and the melting-points and molecular weights of the acids determined. Assuming that no fraction ever contained more than two adjacent homologues, the amounts of the various fatty acids present were calculated from the results arranged in Table I.

Table I.

					Weight of each fatty acid present			
Fraction	B.P. at 2 mm. (°) up to	Weight of acids g.	M.P. (°)	Mol. weight	Myristic g.	Palmitic g.	Stearic g.	"Arachidic" g.
1	136	1·8	53·5–55·5	249·5	0·4	1·4	—	—
2	141	5·6	57·0–59·0	251	1·0	4·6	—	—
3	141	20·6	58·5–59·5	255·5	0·4	20·2	—	—
4	145	18·4	60·0–61·0	258	—	17·1	1·3	—
5	153	27·7	55·0–56·0	263	—	20·8	6·9	—
6	160	18·6	56·0–57·0	269·5	—	9·6	9·0	—
7	165	75·8	68	285	—	—	73·1	2·7
8	167	13·6	68·5–69·0	286	—	—	12·6	1·0
9	Residue	13·5	62	297	—	—	7·2	6·3
Total		195·6*	—	—	1·8	73·7	110·1	10·0

* This figure indicates a loss of less than 3 % of the total saturated fraction, or 0·9 % of the original mixed acids, and was most probably distributed very evenly over the various fractions.

Although reference is made in the discussion to this method of calculating the composition, two points should be explained at this stage. The acids lower in the series than palmitic have been estimated as myristic because, owing to the solubility of the lead soaps of lower members in alcohol, it is extremely improbable that any acid with less than 14 carbon atoms could ever be found in the saturated fraction from the Twitchell separation. Again, for calculation purposes the acids higher than stearic have been grouped together as "arachidic", although it is fully realised that behenic and lignoceric acids were probably also present in this fraction.

	% of saturated acids	% of original mixed acids
Myristic	0·9	0·3
Palmitic	37·7	12·5
Stearic	56·3	18·6
"Arachidic"	5·1	1·7
Loss	—	0·9
Total	100·0	34·0

Analysis of the unsaturated acids.

Various methods have been applied to the problem of determining the constitution of the unsaturated fraction. These are now described in proper sequence below, the first consisting of fractional distillation, followed by hydrogenation of samples from each fraction as used by Klenk and Schoenebeck [1932].

Distillation followed by hydrogenation. 80·5 g. methyl esters prepared from 77·5 g. unsaturated acids were distilled as usual. After the weights and iodine values of each fraction had been recorded, fractions 1–4 were saponified, traces of unsaponifiable matter removed and the fatty acids isolated. Fraction 1 was not studied further, but the acids from 2, 3 and 4, being semi-solid at room temperature, were subjected to the Twitchell separation and the resulting liquid acids were hydrogenated. 1·0–1·5 g. of the esters from each of the remaining fractions were hydrogenated with palladium black and hydrogen at room temperature and the saturated fatty acids isolated, their molecular weights and melting-points being determined. By assuming once again that only two adjacent homologues were present in any one fraction, the amounts of the different acids could be calculated. The results are summarised in Table II.

Table II.

	Methyl esters				Twitchell separation				Hydrogenated acids						
Fraction	B.P. at 1 mm. (°) up to	Wt. g.	% of total unsaturated acids	I.V.	Description	% of total unsaturated acids	I.V.	Mol. wt.	Mol. wt.	M.P. (°)	% C_{14}	% C_{16}	% C_{18}	% C_{20}	% C_{22}
1	100	0·63	0·8	65·4	Only 0·2g. acids obtained		61	287	—	—	—	—	—	—	—
2 *a*	140	4·42	5·5	80·0	Solid	1·2	4	233	—	—	1·0	0·2	—	—	—
b					Liquid	4·3	104	275	273	55–57	—	1·7	2·6	—	—
3 *a*	152	7·78	9·7	97·3	Solid	1·6	8	257	—	—	—	1·5	0·1	—	—
b					Liquid	8·1	119	281	281	60–62	—	0·9	7·2	—	—
4 *a*	155	9·16	11·4	117·7	Solid	1·0	18	266	—	—	—	0·6	0·4	—	—
b					Liquid	10·4	122	284	288	62–64	—	—	8·9	1·5	—
5	156	10·02	12·5	129·9	,,	—	—	—	291·5	63–65	—	—	9·1	3·4	—
6	160	8·94	11·2	143·6	,,	—	—	—	290	63–65	—	—	8·8	2·4	—
7	161	11·27	14·1	155·2	,,	—	—	—	291	62–63	—	—	10·6	3·5	—
8	165	6·46	8·1	179·4	,,	—	—	—	300	62–63	—	—	3·5	4·6	—
9	170	5·89	7·4	222·0	,,	—	—	—	312	64–66	—	—	—	7·4	—
10	175	2·67	3·3	234·2	,,	—	—	—	315	65–67	—	—	—	2·9	0·4
11	Residue	12·80	16·0	183·8	,,	—	—	—	332	64–66	—	—	—	4·6	11·4
Total		80·04	100·0	—	—	—	—	Saturated acids (total)			1·0	2·3	0·5	—	—
								Unsaturated acids (total)			—	2·6	50·7	30·3	11·8

For comparison with these results the following theoretical figures are quoted. For the methyl esters of palmitoleic, oleic and linoleic acids the iodine values are 95, 86 and 173; for methyl esters of C_{20} acids, having 1, 2, 3, 4 and 5 double bonds, they are 78, 158, 238, 319 and 402; for methyl esters of C_{22} acids with 2, 3, 4 and 5 double bonds the corresponding figures are 145, 219, 294 and 369. The molecular weights of the fully hydrogenated C_{16}, C_{18}, C_{20} and C_{22} acids are 256, 284, 312 and 340 respectively.

The conclusions which may be drawn from the results set out in Table II are as follows:

1. The result of the Bertram analysis already mentioned has been very well confirmed in that the unsaturated fraction from the Twitchell separation yielded

on fractional distillation 3·8 % of saturated acids (fractions 2*a*, 3*a* and 4*a*). These obviously consisted of palmitic acid, associated with smaller amounts of stearic acid and lower homologues.

2. From a consideration of the melting-points of the hydrogenated fractions, it is clear that no liquid saturated acid could have been present in the original mixture, a fact which cannot be definitely proved at present, except by hydrogenation experiments.

3. The unsaturated acids contained a small amount of lower homologues, which, estimated as palmitoleic acid, amounted to 2·6 % of the total liquid fraction (fraction 2*b* and 3*b*). This finding was confirmed later.

4. Although the assumption that the hydrogenated fractions each contained only two adjacent homologues has yet to be discussed, the principle as used in the present instance indicated that the mixed unsaturated acids were made up of C_{18} compounds to the extent of approximately 50 %, the major part of the remainder consisting of C_{20} and C_{22} acids; while from the iodine values of the higher fractions and the molecular weights of their hydrogenated derivatives, it seemed most probable that the C_{20} and C_{22} acids contained 2, 3 and 4 double bonds.

This last conclusion is of particular interest, for in their work on octadecenoic acids of pig liver, Channon *et al.* [1934, 1] oxidised portions of these liquid acids by methods which were known to give extremely good yields of hydroxy-derivatives, if applied to pure samples of oleic and linoleic acids. Yet their results suggested that the C_{18} acids did not amount to more than about 28 % of the unsaturated fraction, whereas the present experiment suggests the presence of 50 % of such acids. It has already been stated by Lapworth and Mottram [1925] that their method of oxidation gives considerably reduced yields of hydroxy-derivatives if more highly unsaturated acids are present. It was accordingly decided to distil a large amount of methyl esters prepared from the liquid acids and to oxidise samples of the various fractions obtained. By this means it was hoped to segregate the highly unsaturated acids into the higher fractions and so obtain more representative yields of the hydroxy-derivatives from the C_{18} acids in the lower-boiling fractions.

At the same time the opportunity was taken of investigating further the small amounts of saturated and palmitoleic acids already mentioned as being present in the liquid mixture from the Twitchell separation.

Distillation and subsequent oxidation. 208 g. methyl esters were distilled *in vacuo* into eight fractions. The acids from the first four of these were treated by the modified Twitchell process, and then samples of the liquid acids from all the fractions were oxidised by Lapworth and Mottram's method [1925], a second oxidation being carried out in each case on the petroleum-soluble products to ensure that reaction was complete. From each fraction, in addition to the usual di- and tetra-hydroxy-derivatives, there was obtained a certain amount of fatty acid which was soluble in light petroleum, and which, although virtually saturated, was yet only semi-solid at room temperature. Although this substance has not so far been investigated, the yield obtained from each fraction has been recorded, together with its molecular weight, in order to allow calculations to be made should future work explain its occurrence and origin. It is worthy of note that its molecular weight increased with the molecular weight of the acids from which it was derived. The possibility of this substance being unoxidised material is excluded by the fact that it no longer possessed any appreciable iodine value, while the results both of the Bertram oxidation and of the distillation-hydrogenation experiments definitely exclude the possibility that this peculiar product pre-

Table III.

							Oxidation											
		Distillation					Dihydroxystearic acid					Tetrahydroxystearic acid			Semi-solid petroleum-soluble acids		
Fraction	Description	B.P. at 2 mm. (°) up to	Wt. of acids g.	Mol. wt.	I.V.	% of total unsaturated acids	Wt. oxidised g.	Wt. g.	Mol. wt.	M.P. (°)	% of total unsaturated acids	Wt. g.	M.P. (°)	% of total unsaturated acids	Wt. g.	Mol. wt.	% of total unsaturated acids
1	Solid	140	0·8	213·5	4·9	0·5*	—	—	—	—	—	—	—	—	—	—	—
	Liquid		1·7	248·5	69·4	1·0*	1·508	0·31	300	118–122	0·2	None	—	—	0·39	246	0·3
2	Solid	150	1·6	237	4·2	0·9*	—	—	—	—	—	—	—	—	—	—	—
	Liquid		2·7	266	87·2	1·5*	1·134	0·60	303	118–122	0·8	None	—	—	0·33	267	0·4
3	Solid	160	1·3	252	4·5	0·7*	—	—	—	—	—	—	—	—	—	—	—
	Liquid		3·4	277	87·3	2·0*	2·014	0·76	310	120–123	0·8	0·07	165	0·1	0·60	289	0·6
4	Solid	170	3·7	254	13·7	1·9*	—	—	—	—	—	—	—	—	—	—	—
	Liquid		38·0	280	103·3	19·2*	2·064	0·97	315	127	9·0	0·14	165	1·3	0·35	290	3·3
5	Liquid	175	16·2	280	110·1	8·1	2·489	1·13	319	126–128	3·6	0·22	165	0·7	0·35	289	1·1
6	Liquid	195	40·0	290	132·2	20·0	2·370	0·72	319	127–128	6·0	0·25	160	2·1	0·31	310	2·6
7	Liquid	195	22·5	304	183·3	11·2	2·139	0·30	317	122–123	1·6	0·025	Lost	0·1	0·32	333	1·7
8	Liquid	Residue	32·0	344	200·0	16·0	11·97	None	—	—	—	None	—	—	2·74	336	3·7
Total	—	—	163·9	—	—	83·0	—	—	—	—	22·0	—	—	4·3	—	—	13·7

* These figures have been corrected for small amounts removed after distillation and before Twitchell treatment for determination of iodine values and molecular weights.

existed as such in the liquid acids. That strange by-products are formed by this mild oxidation of these complex mixtures was indicated in every fraction by the fact that a certain amount of unsaponifiable matter was also isolated from the oxidation products, whereas all such matter had been removed from the acids as a whole before their analysis was begun. The results of these distillation-oxidation experiments are set out in Table III.

Several interesting facts should be observed. In the first place only 83 % of the original acids was recovered. The loss of 17 % of the material was due to much decomposition which took place in the undistilled residue, yielding by-products insoluble in light petroleum and not recorded in the table. This fractionation of 208 g. methyl esters was a much longer process than that previously carried out on 80·5 g. (Table II), and this fact, combined with the slightly higher pressure and correspondingly higher boiling-points, accounted for the bulk of the loss. Nevertheless, it may be assumed that the decomposition and consequent loss in iodine value which it necessarily entailed took place entirely in the undistilled residue, in which fraction the most highly unsaturated and therefore the most unstable acids would occur. It may therefore be surmised that as far as oleic, linoleic and palmitoleic acids are concerned, the quantitative results remain unaffected.

With regard to the hydroxy-derivatives, their melting-points are recorded in Table III and are typical for the usual products obtained by this means before purification by fractional crystallisation, while the molecular weights quoted for the dihydroxystearic acid from fractions 5, 6 and 7 approximate closely to the theoretical value of 316. It is of importance to observe that oleic acid was shown by these results to be present in all the fractions except the last, and that linoleic acid was detected in all but numbers 1, 2 and 8. The bearing of this finding on the distillation method of analysis as applied to unsaturated fatty acid mixtures is discussed later.

Palmitoleic acid. The presence of palmitoleic acid in the unsaturated fraction, which was already suggested by the results in Table II, was confirmed in the present instance by the molecular weights and melting-points of the dihydroxy-derivatives from fractions 1, 2 and 3. When the first of these was analysed, the values obtained were C, 67·3; H, 11·0 %; mol. wt., 300. For an equimolecular mixture of dihydroxypalmitic and dihydroxystearic acids, the corresponding figures should be 67·5, 11·35 % and 302. It is therefore probable from both experiments that the content of palmitoleic in the unsaturated acids was 2–3 %.

The saturated acids from the unsaturated fraction. The Bertram oxidation and the results of Tables II and III all suggest that the saturated acids still remaining in the liquid fraction amounted to approximately 3·8 %. In order to analyse these, each of the solid portions obtained in the last experiment was fractionally recrystallised from acetone and its nature investigated.

From fractions 3 and 4 the solid acids (mol. wt. 252 and 254), consisted mainly of palmitic acid. They both gave crops of crystals from acetone, melting between 57·5 and 61·5° and typical C and H figures of C, 74·5; H, 12·5 %. ($C_{16}H_{32}O_2$ requires C, 74·9; H, 12·6 %; mol. wt., 256.)

After recrystallisation, fraction 2, which had mol. wt. 237, melted at 54–56° and therefore most certainly contained palmitic and myristic acids. Fraction 1 (mol. wt. 213·5) gave three crops and a mother-liquor, the details of which are shown in Table IV.

(Required for $C_{10}H_{20}O_2$: C, 69·8; H, 11·6 %; for $C_{12}H_{24}O_2$: C, 72·0; H, 12·0 %; and for $C_{14}H_{28}O_2$: C, 73·7; H, 12·3 %.) It must be concluded from these experiments that this small amount of solid acid, which is not separated in the saturated fraction in the Twitchell method, but is estimated by a Bertram oxidation on the unsaturated fraction, consisted mainly of palmitic with small quantities of myristic, lauric and possibly *n*-decanoic acids. Table II also suggested the presence of stearic acid in small amounts.

Table IV.

Crop	Weight (g.)	M.P. (°)	C %	H %	Probable constitution
1	0·11	55·5–57·5	74·2	12·65	Mainly palmitic acid
2	0·08	45 –46	—	—	Lauric mixed with myristic acid
3	0·18	46	72·4	12·1	
Mother-liquor	0·40	—	69·7	11·5	Possibly *n*-decanoic acid present

The detection of highly unsaturated acids by oxidation. As shown in Table III, 11·97 g. of the undistilled residue were oxidised by the usual method. After extraction with ether, the resulting aqueous solution (12 l.) was evaporated to 700 ml. and the residue allowed to cool. Much inorganic material crystallised out along with small amounts of organic acids and some tar. These were filtered off and the residue extracted three times with boiling absolute alcohol. 0·3 g. of a crude mixture was obtained and was fractionally crystallised from aqueous alcohol and from water after treatment with charcoal. The first crop (0·04 g.) had M.P. 200°, but the major part, even after exhaustive attempts at purification, melted constantly at 186° and gave on analysis C, 55·2; H, 9·35 %. The mineral salts were then extracted three times with boiling 70 % alcohol, and 0·6 g. of a very impure organic acid was isolated. By repeated recrystallisations from both water and aqueous alcohol after charcoal treatment, 0·2 g. of a pure product was prepared, M.P. 195°. (Found: C, 54·5; H, 9·1 %. Required for $C_{20}H_{40}O_{10}$: C, 54·5; H, 9·1 %; and for $C_{22}H_{44}O_{10}$: C, 56·4; H, 9·5 %.) It therefore seems most probable from these figures that the fraction extracted by absolute alcohol was a mixture of octahydroxyarachidic and octahydroxybehenic acids, while that isolated by 70 % alcohol was the former of these alone. Owing to the large amounts of mineral salts and the solubility of these hydroxy-compounds, the extraction cannot be regarded as in any way complete. The experiment does show however that, in addition to the usual arachidonic acid first discovered by Hartley [1909], there is probably also present in liver fat the corresponding C_{22} acid with four double bonds.

Bromination experiments. All the known unsaturated acids form soluble liquid bromo-derivatives, in addition to solid isomerides, and usually the former can neither be identified nor estimated. This explains why bromination has never been regarded as a satisfactory method of analysing fatty acid mixtures. New interest in such a process has recently been stimulated however by the work of Ault and Brown [1934] who have suggested a method, to which reference is made later, for estimating arachidonic acid by this means.

50·3 g. of the unsaturated acids were therefore brominated in ether at 0° in the usual way. The insoluble product was filtered off and washed with ether and then excess bromine removed from the filtrate and washings by sodium thiosulphate solution. On removing the ether and taking up the residue in light petroleum (B.P. 80–100°) and leaving at 0° for one night, a further small amount of insoluble bromo-derivative crystallised out. This was filtered off, the filtrate taken to dryness, dissolved in light petroleum (B.P. 40–60°) and kept at 0° for some days, when crystalline tetrabromostearic acid was deposited. The results are summarised in Table V, where it should be noted that the saturated acids in the unsaturated fraction are taken as being 4 % (the nearest unit) and the true data for the petroleum-soluble bromides appropriately corrected for that amount.

Table V. *Bromination of 50·3 g. unsaturated acids.*

No.	Description	Wt. g.	M.P. (°)	Bromine %	Corresponding wt. of unsaturated acids g.	Original unsaturated acids %	Calculated I.V. of these acids
1	Insoluble in ether	13·2	240 indef.	68·3	4·18	8·3	342
2	Insoluble in high-boiling petroleum	1·75	,,	68·0	0·56	1·1	337
3	Tetrabromostearic acid	2·0	112–113	53·3	0·93	1·8	181
4	Soluble in cold petroleum	77·0	—	45·0	42·4	84·3	130
5	Saturated acids	2·0	—	—	2·0 (sat.)	4·0	—
	Total accounted for				50·0	99·5	

The absence of linolenic acid. Normally when linolenic acid is present in a mixture, it yields a hexabromostearic acid soluble in benzene, insoluble in ether and melting at 180°. To search for this substance, 2·9 g. of the ether-insoluble products of bromination (Table V), were thoroughly extracted with benzene, but the two fractions so obtained melted at the same temperature (240° with decomposition). The absence of linolenic acid was further confirmed by the oxidation experiment already described in which octahydroxyarachidic acid was isolated (p. 1364), for in that case no trace of hexahydroxystearic acid could be found. It is therefore safe to conclude that, if this unsaturated acid were actually present, the amount must have been extremely small.

Apart from this result, the bromination experiment added little to the information already gained. It confirmed the presence of ordinary linoleic acid and of higher unsaturated acids of the C_{20} or C_{22} type, but unfortunately with such a complex mixture nothing of any value could be concluded from the soluble bromide fraction (Table V, No. 4), which constituted such a large proportion of the whole. The minimum amount of highly unsaturated C_{20} and C_{22} acids present can however be stated from fractions 1 and 2 to have been 9·4 % of the total unsaturated fraction.

DISCUSSION.

The general analysis just described has confirmed and extended the preexisting knowledge of the fatty acids contained in mammalian livers and has in several aspects tested the methods by which they may be investigated.

The relative values of the Twitchell and Bertram processes were well illustrated. There is no doubt that the lead soap separation as modified by Hilditch and Priestman [1931] gave an exceedingly good separation of the saturated and unsaturated acids, whilst the small amount of saturated compounds still remaining in the liquid portion was readily estimated by a Bertram oxidation, carried out on the liquid acids rather than on the original mixture. The value so found for these residual acids was confirmed twice by distillation experiments.

The method of calculating the amounts of different acids present by distillation of the esters, followed by estimations made on the assumption that each fraction never contains more than two homologues, has been much used by

Hilditch and his co-workers and has been recently discussed in detail by Hilditch [1934]. Provided that a sufficiently large number of fractions be taken, it is most improbable in the case of the saturated acids alone that any one of the fractions will contain more than relatively small traces of a third homologue. Even in a case, such as the one described in the present communication, where only nine fractions were taken, the final results calculated on this basis probably approximated to within a few units of the true value in the large figures recorded. At the same time it is difficult to assume a greater degree of accuracy than this, for even if it be allowed that the presence of a third homologue be negligible, other small but appreciable errors may arise, as for example in determining the mean molecular weight of each fraction, a figure on which the whole process of deducing the composition depends. Provided these and other small errors at this stage neutralise each other, as they may often do, the conclusions will approximate closely to the true values; but if on the other hand they happen to accumulate, the figures finally arrived at for the main constituents will only approach within a few units of the actual percentages which a more accurate method would yield. Unfortunately a more accurate method for this purpose has not yet been devised.

The liquid acids present a still more difficult problem. In the present case the initial mixture of unsaturated acids was too complex for proper analysis to be made by fractional distillation. The failure of the process for this purpose was shown by the fact that oleic and linoleic acids were proved by oxidation methods to be present in the majority of the fractions, even though the boiling points of these differed in the extreme by as much as 35°. Again distillation followed by hydrogenation made it certain that C_{20} and C_{22} acids, having two, three and four double bonds also existed in the same fractions. Consequently it was impossible to elucidate satisfactorily the composition of the unsaturated liver acids by simple calculation from such fractions, although such a procedure may well be applicable to the less complex fatty acids of the glycerides and phosphatides from plant sources.

Hydrogenation, therefore, as applied by Klenk and Schoenebeck [1932] appears to be the most satisfactory approach so far devised for the analysis of such mixtures, the actual hydrogenation either preceding or following the fractional distillation. Such a method eliminates the danger of overlooking the presence of liquid saturated acids and, taken in conjunction with the other data acquired, it goes far towards yielding a true estimate of the various constituents. For the acids under discussion it has shown better than any other method the relative amounts of C_{16}, C_{18}, C_{20} and C_{22} compounds and has suggested their relative degrees of unsaturation. The figures so obtained for the C_{20} and C_{22} acids, which must have had two or three double bonds, was surprisingly large, but they have confirmed very closely the corresponding values found by Klenk and Schoenebeck in their work on the acids from ox liver. Unfortunately it has been impossible to prepare any derivatives by which these compounds might be characterised. The same method gave the content of C_{18} acids as approximately 50 % of the liquid portion and, from the yields of di- and tetra-hydroxy-derivatives, whether obtained by oxidising the total acids as in Part I of this series [1934, 1] or each of the various fractions resulting on distillation, it may be assumed that the quantity of oleic acid was about five times greater than that of the linoleic acid. The amounts of these acids in the unsaturated fraction should therefore have been of the order of 42 and 8 % respectively.

In at least two ways the presence of a lower unsaturated acid, which was most probably palmitoleic, was proved.

On investigating the saturated acids still remaining in the liquid fraction and obtained from it on distillation, the presence of lauric, myristic and possibly *n*-decanoic acids was shown, in addition to palmitic and traces of stearic acids. According to the theory of β-oxidation of fatty acids, these lower even-numbered members might well be expected to appear in small amounts in the liquid fraction of the liver acids. Any acids having less than 10 carbon atoms would have been previously removed in the preparation of the total acids from the liver, owing to their solubility in water or their capacity to distil readily in steam.

With regard to the true amount of arachidonic acid present, Ault and Brown [1934] have prepared pure arachidonic acid in different ways and brominated the pure product. They found that no matter how the acid was made, 1 g. on the average yielded approximately 0·842 g. of polybromide, instead of the theoretical value of 3·1 g. They then assumed that on brominating a mixture of acids this proportion of ether-insoluble polybromide would remain the same and so they suggested the following formula:

$$\%\text{ of arachidonic acid} = \frac{\text{Weight of ether-insoluble bromide from 100 g. of mixed acids} \times 100}{84{\cdot}2}.$$

When this was applied in the present instance, the content of arachidonic acid became:

$$\frac{13{\cdot}2 \times 100 \times 100}{50{\cdot}3 \times 84{\cdot}2}\,\% = 31{\cdot}2\ \%.$$

Now if it may be assumed for the moment that fraction 4 in Table V consisted entirely of the bromo-derivatives of a C_{20} acid with one double bond, together with the corresponding derivatives of arachidonic acid, the maximum amount of the latter acid which could possibly have been present may be calculated to be 26·5 %. That it must actually have been considerably less than this figure is obvious, for from the distillation-hydrogenation experiment it is certain that the petroleum-soluble fraction (No. 4, Table V) must have contained derivatives of acids all with higher iodine values than that of a C_{20} acid with one double bond, and these would all considerably decrease this estimated maximum for the content of arachidonic acid.

The method of Ault and Brown therefore did not apply in the present case. This might have been due to either of two reasons. In the first place, it is most probable that some highly unsaturated C_{22} acids were present in small amounts in the original mixture, as well as C_{20} compounds other than the ordinary arachidonic acid. These would naturally make it unfair to test the validity of the procedure on such a sample of acids.

In the second place the high value obtained in the present instance by the application of this method might have been due to the fact that in the presence of other unsaturated acids the proportion of arachidonic acid yielding a solid ether-insoluble bromide is greatly enhanced. Such an explanation is possible in the light of the work of Rudy [1932], who found that on brominating acids of this type the relative proportions of the different forms produced varied considerably with such factors as the type of solvent and the speed of bromination. That they may also vary according to the type and proportions of the other acids present in the mixture seems very possible.

The following table shows a summary of the approximate composition of the pig liver fatty acids as far as it may be deduced from these experiments. From the discussion as a whole it is clear that the figures cannot be other than approximate.

	Saturated acids %	Original mixture %
n-Decanoic and lauric acids	1	0·4
Myristic acid	2	0·7
Palmitic acid	39	14·0
Stearic acid	53	18·8
"Arachidic" acid	5	1·7
Total	100	35·6

	Unsaturated acids %	Original mixture %
Palmitoleic acid	2·5	1·5
Oleic acid	44	28
Linoleic acid	8	5
Linolenic acid	Absent	—
C_{20} acid	31	20
C_{22} acid	12	7·5
Total	97·5	62·0

Total loss – 2·4 %.

The C_{20} and C_{22} acids probably existed in the mixture as compounds having from two to four double bonds.

SUMMARY.

The pig liver fatty acids have been analysed by bromination and distillation experiments, the latter being followed both by oxidation and by hydrogenation processes. The results are discussed in full and summarised immediately above.

The authors desire to express their thanks to Prof. H. J. Channon for his continual interest and encouragement during the course of this work.

REFERENCES.

Anderson (1929). *J. Biol. Chem.* **83**, 169.
Ault and Brown (1934). *J. Biol. Chem.* **107**, 615.
Bertram (1927). *Chem. Weekblad*, **24**, 226, 319.
Bosworth and Brown (1933). *J. Biol. Chem.* **103**, 115.
—— and Sisson (1934). *J. Biol. Chem.* **107**, 489.
Channon, Irving and Smith (1934, 1). *Biochem. J.* **28**, 840.
—— —— —— (1934, 2). *Biochem. J.* **28**, 1807.
Hartley (1909). *J. Physiol.* **38**, 353.
Hilditch (1934). *Biochem. J.* **28**, 779.
—— and Priestman (1931). *Analyst*, **56**, 364.
Klenk and Schoenebeck (1932). *Z. physiol. Chem.* **209**, 112.
Lapworth and Mottram (1925). *J. Chem. Soc.* **127**, 1628.
Rudy (1932). *Z. physiol. Chem.* **210**, 236.
Spielman (1934). *J. Biol. Chem.* **106**, 87.
Turner (1931). *Biochem. J.* **25**, 49.

CLXVI. THE ALGAE.

I. THE LIPOCHROMES OF *FUCUS VESICULOSUS*.

BY ISIDOR MORRIS HEILBRON AND
RICHARD FRANCIS PHIPERS.

From the Chemical Department, University of Manchester.

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THE earliest recorded investigation of the fat-soluble pigments of the brown algae was made by Cohn [cited by Kylin, 1912], who isolated from the Phaeophyceae one brown pigment, phaeophyll, which he presumed was closely related to chlorophyll. Millardet [1869] clarified the position by showing that phaeophyll could be resolved by partition processes between light petroleum and alcohol into chlorophyll, a yellow lipochrome, phycoxanthin, and a brown water-soluble pigment, phycophain. Sorby [1873] came to the conclusion that the Fucaceae, a branch of the Phaeophyceae, contained three different yellow lipochrome pigments, xanthophyll, phycoxanthin (which he renamed fucoxanthin) and lichnoxanthin, the last occurring in very small amounts. Reinke [1876] considered that Sorby's method of distinguishing between the yellow pigments was unreliable and was himself only able to detect fucoxanthin, which was not identical with the xanthophyll of the higher plants. Tswett [1906], employing the chromatographic method, confirmed the presence of carotene first demonstrated by Tammes [1900] and also of fucoxanthin, the presence of which had recently been denied by Gaidukow [1903]. Tswett also distinguished a further lipochrome pigment, fucoxanthophyll, and showed that whereas fucoxanthin develops an intense royal blue colour with hydrochloric acid, fucoxanthophyll gives only a green colour. Kylin [1912] further defined the colour reactions of fucoxanthin and stated that fucoxanthophyll was identical with the xanthophyll of the higher plants (which gives no colour with hydrochloric acid). Willstätter and Page [1914] isolated fucoxanthin as a crystalline compound from various Phaeophyceae and assigned to it the formula $C_{40}H_{54}O_6$ or $C_{40}H_{56}O_6$. By partition processes between aqueous methyl alcohol, ether and light petroleum, they further obtained a pigmented solution which gave no colour with hydrochloric acid and on these grounds concluded that "xanthophyll" was also present. These authors observed that it was apparently impossible to obtain fucoxanthin from brown algae after a period of 10 days had elapsed from the time of gathering; they did not, however, pursue this matter further.

In view of the wide disparity in the results of the earlier investigators we have again carried out an examination of the lipochromes present in *Fucus vesiculosus* and have employed for this purpose both the fresh alga and old dried material. Our results show that whereas fresh *Fucus vesiculosus* contains the characteristic brown algal pigment fucoxanthin together with carotene, the former pigment is wholly absent from the dried material, which contains in its place zeaxanthin. In addition a constitution for fucoxanthin based on its chemical properties, and permitting of such a post-mortal transformation, is discussed later in the paper.

EXPERIMENTAL.

Old dry Fucus vesiculosus. The material, collected during the summer months from the north coast of Scotland, was air-dried over a prolonged period and milled, giving a pale fawn powder which was subjected to a systematic solvent extraction, a typical experiment being outlined below [see also Heilbron *et al.*, 1934]. The powder (36 kg.) was intermittently stirred with 95 % ethyl alcohol (40 l.) for 18–24 hours at room temperature. The deep olive-green solution obtained by filtration through an edge filter was diluted with water (10 l.) and shaken with successive portions of light petroleum (B.P. 40–60°) until the extracts were practically colourless. The latter were combined and concentrated under nitrogen (extract A). The alcoholic phase, after further dilution with an equal volume of water, was completely extracted with ether, the ethereal solution washed with water, dried and concentrated to about 2000 ml. (extract C). The residual meal was extracted with ether (40 l.), the ethereal solution furnishing extract B.

Extract C. This was dried by distillation with an equal volume of benzene (final volume 400 ml.) and the cold benzene solution adsorbed in four portions on columns (50 × 5 cm.) filled with Merck standardised Brockmann alumina (1 part) and ordinary alumina (1 part), the chromatogram being developed with light petroleum (B.P. 40–60°); three distinct bands were thus obtained.

The uppermost band (deep olive-green), which could not be completely eluted with methyl alcohol, was desorbed slowly with a mixture of water, methyl alcohol and pyridine. The pigment, obviously of the chlorophyll type (probably allomerised chlorophyll), was not further examined.

Elution of the second band (deep brick-red, immediately below the green band) was performed with methyl alcohol; the solution was concentrated to small bulk, poured into water and extracted with ether. The washed and dried ethereal solution was concentrated (2 ml.) and diluted with light petroleum. After standing in the ice-chest for 24 hours, the precipitated pigment was repeatedly crystallised from benzene-methyl alcohol, from which it separated in deep red glistening needles, M.P. 211° (uncorr.). It was characterised as zeaxanthin by its absorption maxima in CS_2 (518, 483 and 451 $m\mu$) and by analysis. (Found: C, 83·2; H, 10·1 %. $C_{40}H_{56}O_2$, 0·5 CH_3OH requires C, 83·2; H, 10·2 %) [*cf.* Kuhn and Grundmann, 1934].

The third pigmented section, formed at the base of the column, after elution with methyl alcohol and removal of solvent yielded a pale yellow mobile oil from which no crystalline pigment could be obtained. This zone consisted of terpenoid substances previously reported by Heilbron *et al.* [1934].

Extracts A and B. After removal of solvent the residue in each case was saponified with 10 % methyl alcoholic potash (3 l.) at 40° for 3 hours. The unsaponifiable material obtained by ethereal extraction was freed from fucosterol and hentriacontane by crystallisation from methyl alcohol as described by Heilbron *et al.* [1934], and the deep red methyl alcoholic mother-liquors were steam-distilled in an atmosphere of nitrogen to remove volatile terpenes. The residue was extracted with benzene and the dried concentrated solution (50 ml.) chromatographically resolved as described above. The completed chromatogram showed three distinct red bands, the uppermost of which on elution with methyl alcohol yielded a further quantity of zeaxanthin, M.P. 208–209°. The central band was also eluted with methyl alcohol and the solution concentrated until turbid. The pigment which separated on cooling was recrystallised from benzene-methyl alcohol, giving beautiful metallic flakes, M.P. 178–179° (uncorr.), of β-carotene; absorption maxima in CS_2, 518, 483 and 451 $m\mu$. (Found: C, 89·3, H, 10·8 %. $C_{40}H_{56}$ requires C, 89·5; H, 10·5 %.)

Elution of the brownish red zone at the base of the column provided a further quantity of terpenoid material.

Fresh Fucus vesiculosus. The minced alga (50 kg.) was freed from mucilages by washing twice with aqueous acetone (50 l. of 40 %) as described by Willstätter and Page [1914]. The washed material was covered with methylated spirit (50 l.) and allowed to stand for 24 hours with intermittent agitation. The alcoholic extract was resolved as described above into extracts A, B and C.

Extract C. This was dried with benzene and the solution (3 l.) chromatographically resolved in 15 portions in columns (50 × 5 cm.) filled with partially deactivated alumina (prepared by washing Brockmann activated alumina with methyl alcohol and subsequent drying in air) the chromatograms being developed with benzene-light petroleum (1:1) and finally washed with light petroleum (B.P. 40–60°)[1]. The completed chromatogram formed three definite bands, the uppermost of which, a deep olive-green, appeared to be identical with that obtained from extract C of the old alga. The middle broad crimson band was eluted with methyl alcohol and the deep red solution diluted with an equal volume of water and allowed to stand, atmospheric oxygen being excluded by a thin layer of light petroleum. The pigment which slowly separated as a semi-crystalline precipitate at the interphase was dried *in vacuo* over sodium sulphate. On recrystallisation from ether-light petroleum pure fucoxanthin separated in deep orange-red needle aggregates, M.P. 166–168° (uncorr.); absorption maxima in carbon disulphide, 510, 477 and 445 $m\mu$; in chloroform 487 and 456 $m\mu$. (Found: C, 75·3; H, 9·3 %.) Karrer *et al.* [1931] give the M.P. as 160° (corr.) and maxima in chloroform at 492 and 457 $m\mu$. Willstätter and Page [1914] record a M.P. at 159·5–160·5° (corr.) and 158° (uncorr.). In agreement with these latter authors we have observed that whereas the blue colour produced by the action of acids on fucoxanthin exists in aqueous solution only in the presence of ether, when a light petroleum solution is treated with 25 % hydrochloric acid the blue colour remains entirely in the upper layer.

The third band on elution with methyl alcohol gave an orange-red solution, which in contrast to that obtained above, yielded its contained pigment to light petroleum on dilution. When a test portion of this was treated with 25 % hydrochloric acid a magenta colour was observed in the acid layer in addition to the royal blue colour in the light petroleum. The solution, obviously complex, was ultimately resolved by adsorption on fuller's earth, two distinct pigments being obtained. That occurring in the larger proportion on recrystallisation from ether-light petroleum yielded fucoxanthin, whilst the other (*circa* 2 mg.), which was extremely resinous, has not as yet been obtained in a crystalline state. Its ethereal solution gives with 25 % hydrochloric acid a colourless upper and a magenta lower layer and in carbon disulphide shows general absorption with a marked inflection at 500 $m\mu$. The pigment is presumably the "fucoxanthin-β" referred to but not isolated by Kylin [1927], who stated that there was a small amount of another pigment giving a more violet colour with hydrochloric acid.

We are inclined to believe that it is partially oxidised fucoxanthin, a view supported by catalytically hastening the autoxidation [*cf.* Palmer and Thrun, 1916] of the pure pigment. A solution of fucoxanthin (25 mg.) in glacial acetic acid (10 ml.) was heated on a steam-bath for 1 hour with ferric chloride (10 mg.) and the cooled solution rendered alkaline with dilute sodium hydroxide solution.

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[1] If fully activated alumina is used the pigments are so strongly adsorbed that separation of the zones is impossible.

The pigment was removed with ether and the washed and dried ethereal solution evaporated. The residue was dissolved in benzene (25 ml.) and chromatographically examined on a column of activated alumina, when only one strongly adsorbed red band was obtained. Elution with methyl alcohol yielded a red solution from which no crystallisable material could be obtained. The pigment contained in this eluate was, however, identical with "fucoxanthin-β" in that treatment of an ethereal solution with 25 % hydrochloric acid gave a magenta-coloured acid layer and in CS_2 it showed a pronounced inflection at $502 m\mu$.

Extract A. This was examined in detail in order to ascertain whether any "xanthophyll" other than fucoxanthin was present. The concentrated light petroleum solution (3000 ml.) was directly resolved by adsorption in 12 portions on partially deactivated alumina, giving a complex chromatogram showing five distinct bands (Fig. 1).

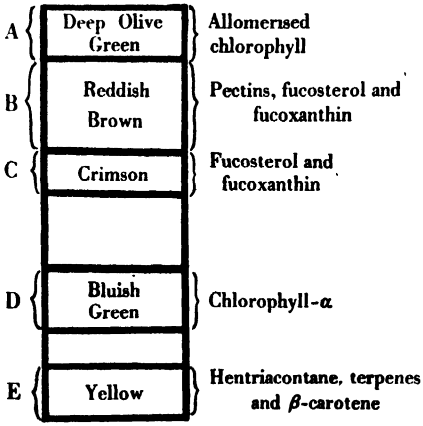
A
Deep Olive Green
Allomerised chlorophyll
B
Reddish Brown
Pectins, fucosterol and fucoxanthin
C
Crimson
Fucosterol and fucoxanthin
D
Bluish Green
Chlorophyll-α
E
Yellow
Hentriacontane, terpenes and β-carotene

Fig. 1.

Band B. This band was eluted with methyl alcohol and the eluate diluted to 75 % with water and extracted twice with light petroleum. The lower phase, after separation and further dilution, yielded precipitated fucoxanthin, M.P. 161–163°. The washed light petroleum phase was dried and a test portion evaporated, dissolved in ether and treated with 25 % hydrochloric acid; a green lower layer (finally changing to blue) was thus obtained (agreement with Tswett's [1906] fucoxanthophyll). This solution should, however, according to Willstätter and Page [1914] contain "xanthophyll". The total light petroleum solution was consequently evaporated and the residue treated with cold methyl alcohol; a quantity of brown pectinoid material (*circa* 2 g.) was precipitated[1]. The mother-liquors were evaporated, the residue was dissolved in the smallest volume of ether and a large excess of light petroleum added. After standing for 24 hours, the practically colourless light petroleum solution (which was found to contain fucosterol, M.P. 124°) was decanted from the deep red crust, which, on recrystallisation from ether-light petroleum, yielded pure fucoxanthin, M.P. 166–167° (uncorr.). It is thus evident that the green "acid" colour is not due to any specific pigment such as fucoxanthophyll but must be mainly fucoxanthin contaminated with the above materials.

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[1] When using alga gathered in the winter months we have found that the amount of this substance is so large as to render the isolation of any pigment exceptionally difficult.

Band C. The bright red band on elution and resolution by the methods just described furnished fucoxanthin and fucosterol.

Using the methods described the amounts of fucoxanthin and zeaxanthin were 1–1·5 g. and 2 g. respectively.

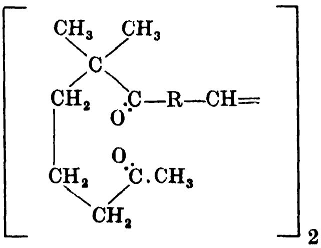
Fucoxanthin.

Although the analytical values clearly reveal the presence of six oxygen atoms, Zerewitinow determinations on the pure pigment indicate that only four of these exist as hydroxy-groups. The following determinations were kindly performed for us by Drs Roth and Möller of Heidelberg:

6·74 mg. fucoxanthin 0·93 ml. CH_4 (N.T.P.) : 3·9 active H.
6·478 mg. „ 0·92 ml. „ 4·0 „

Karrer *et al.* [1931] have stated that fucoxanthin was optically active ($[\alpha]_{Cd}+73\cdot5°$ in chloroform) but a re-investigation of our higher-melting pigments shows that it is inactive ($\alpha^{20}_{Cd} \pm 0\cdot02°$ in benzene) ($c=0\cdot062$; $l=1$). This finding presents a striking parallel with the results obtained by Kuhn and Grundmann [1934], who by purification processes raised the melting-point of zeaxanthin from 207° to 215° and likewise found that the reported optical activity ($[\alpha]_{Cd}-70°$) of this pigment was non-existent.

Repeated attempts have been made, without avail, to demonstrate the presence of carbonyl groups in the pigment, and thus, if such exist, they cannot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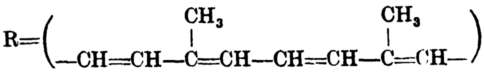
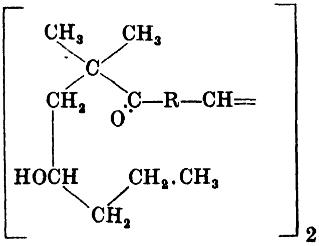

$$R=\left(-CH=CH-\overset{CH_3}{\overset{|}{C}}=CH-CH=CH-\overset{CH_3}{\overset{|}{C}}=CH-\right)$$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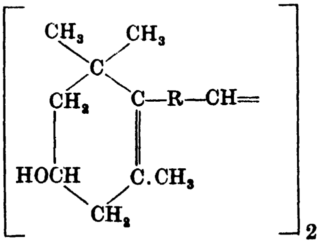
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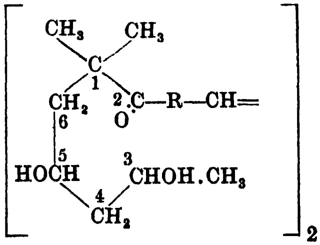
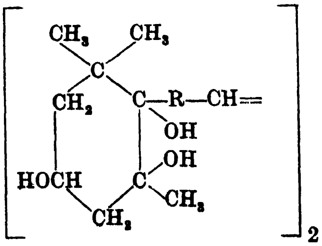

IV


V

occupy positions analogous to those in astacene [Karrer and Loewe, 1934] or rhodoxanthin [Kuhn and Brockmann, 1933]. This does not, however, preclude the possibilities of such groups actually being present, for β-carotenone (I) [Kuhn and Brockmann, 1932] only yields a dioxime, and capsorubin (II) which according to Zechmeister and v. Cholnoky [1935] is a dihydroxyketone fails altogether to form any ketonic derivatives.

The parallel with the inert oxygen atoms in fucoxanthin leads us tentatively to suggest the possibility that this pigment has the structure (IV) (being in effect a dihydroxy-capsorubin[1]). Such a constitution necessitates a formula $C_{40}H_{60}O_6$ in place of the hitherto assumed $C_{40}H_{56}O_6$, and that such an alteration is justified is seen from the following analyses.

	Carbon %	Hydrogen %
$C_{40}H_{56}O_6$	75·9	8·9
$C_{40}H_{60}O_6$	75·4	9·4
Found	75·3	9·3
Found [Karrer *et al.*, 1931]	75·5	9·1

Further, as has already been shown, the structure for fucoxanthin must be such as to accommodate a facile *post mortem* transformation into zeaxanthin (III). Whereas our suggested formula would permit of such a transformation it would be extremely difficult to account for this if fucoxanthin had hydroxy-groups in either positions $C_4 : C_4'$ or $C_6 : C_6'$.

Additional support of (IV) has been unexpectedly obtained by drastic perhydrogenation with an excess of platinum catalyst. The hydrogenation was carried out in acetic acid under an average pressure of 30 lbs./sq. in. The fully hydrogenated product (tetranitromethane test) crystallised from methyl alcohol-ethyl acetate in small plates, M.P. 63–64°, and was proved by analysis to have an oxygen content lower than that of the anticipated perhydrofucoxanthin. (Found: C, 82·3; H, 13·9 %. $C_{40}H_{78}O_2$ requires C,.81·4; H, 13·2 %.) Unfortunately, owing to lack of material we have so far been unable to confirm the above result, but our experiments will be continued as soon as suitable fresh *Fucus vesiculosus* becomes available.

Against formula (IV) Karrer *et al.* [1931] found that only 10 molecules of hydrogen were required to saturate fucoxanthin whereas 13 molecules would be required for a complete conversion into perhydrozeaxanthin. Further, as a product of energetic oxidation these authors only detected αα′-dimethylmalonic acid whereas αα′-dimethylsuccinic acid is also to be anticipated. We intend to reinvestigate these results on our chromatographically purified pigment in due course.

The fact that alcoholic alkali reacts with fucoxanthin to produce a substance much more susceptible to the action of acids was first observed by Kylin [1912] and confirmed by Willstätter and Page [1914]. We have now examined this effect in detail. Fucoxanthin (100 mg.) was allowed to stand for 24 hours with 5 % methyl alcoholic potash (25 ml.) in an atmosphere of nitrogen. The alkaline solution, after dilution, was extracted with ether. The washed and dried solution was evaporated to dryness under nitrogen and the residue dissolved in methyl alcohol. The resultant solution is extraordinarily sensitive to acids, rapidly developing a deep blue colour when exposed to the atmosphere, and this extreme lability has so far precluded attempts at recrystallisation. That the products formed (which for convenience we propose to name generically the *iso*fucoxanthins) are multiple is evidenced by chromatographic resolution from a benzene solution on alumina, when three distinct crimson bands are formed. It is possible to obtain a partial distinction between these components by the effect of $N/10$ acetic acid (acid of this strength being without action on fucoxanthin itself).

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[1] In this connection the recent isolation by Lederer [1934] of capsorubin from the ascidian *Botryllus Schlosseri Pallas* is highly significant.

Band	Action of $N/10$ acetic acid	Absorption maxima in CS_2 $m\mu$	Estimated amount %
A	Slight browning	474, 448, 422	25
B	Intense royal blue	474, 448, 422	60
C	Blue colour slowly develops	475, 449, 420	15

The shift in absorption maxima implies that the number of conjugated double bonds has decreased, and bearing in mind the nature of the reaction it seems possible to us that fucoxanthin (IV) undergoes an aldol condensation, giving rise to (V).

SUMMARY.

Fucus vesiculosus has been exhaustively examined with regard to its lipochromes, and it has been found that whereas the dead material contains β-carotene and zeaxanthin the living plant contains β-carotene and fucoxanthin. These results explain the apparently contradictory statements of the earlier investigators.

No trace of the xanthophylls usually associated with the higher plants could be detected in the fresh alga, and thus zeaxanthin must be a *post mortem* product of fucoxanthin.

A chemical investigation of fucoxanthin shows that the formula of this pigment is probably $C_{40}H_{60}O_6$. The melting point has been raised to 166–168° (uncorr.); the pure pigment, contrary to earlier statements, is optically inactive.

We wish to thank Mr A. E. Gillam of this Department for carrying out spectroscopic determinations and Mr A. Lumley and Dr J. A. Lovern of the Torry Research Station, Aberdeen, for the algal materials. Our thanks are also due to the Department of Scientific and Industrial Research for a grant to one of us (R. F. P.).

REFERENCES.

Gaidukow (1903). *Ber. deutsch. bot. Ges.* **21**, 535.
Heilbron, Phipers and Wright (1934). *J. Chem. Soc.* 1572.
Karrer, Helfenstein, Wehrli, Pieper and Morf (1931). *Helv. Chim. Acta*, **14**, 614.
—— and Loewe (1934). *Helv. Chim. Acta*, **17**, 745.
Kuhn and Brockmann (1932). *Ber. deutsch. chem. Ges.* **65**, 894.
—— —— (1933). *Ber. deutsch. chem. Ges.* **66**, 828.
—— and Grundmann (1934). *Ber. deutsch. chem. Ges.* **67**, 596.
Kylin (1912). *Z. physiol. Chem.* **82**, 221.
—— (1927). *Z. physiol. Chem.* **166**, 39.
Lederer (1934). *Compt. Rend. Soc. Biol.* **177**, 1086.
Millardet (1869). *Ann. Sci. Nat. Bot.* **10**, 59.
Palmer and Thrun (1916). *J. Ind. Eng. Chem.* **8**, 614.
Reinke (1876). *Jahr. wiss. Bot.* **10**, 400.
Sorby (1873). *Proc. Roy. Soc. Lond.* **21**, 474.
Tammes (1900). *Flora*, **87**, 240.
Tswett (1906). *Ber. deutsch. bot. Ges.* **24**, 236.
Willstätter and Page (1914). *Liebig's Ann.* **404**, 237.
Zechmeister and v. Cholnoky (1935). *Liebig's Ann.* **516**, 30.

CLXVII. THE ALGAE.

II. THE RELATIONSHIP BETWEEN CERTAIN ALGAL CONSTITUENTS.

BY ISIDOR MORRIS HEILBRON, EDRYD GWYLFA PARRY
AND RICHARD FRANCIS PHIPERS.

From the Chemical Department, University of Manchester.

(*Received April 6th, 1935.*)

IN a former publication [Heilbron *et al.*, 1934] covering a preliminary examination of the Phaeophyceae a description was given of the isolation of a new sterol, fucosterol, $C_{29}H_{48}O$. We have now extended our investigations to members of the Chlorophyceae and Rhodophyceae in order to ascertain whether this sterol is common to all species of algae and also to determine the character of the lipoid pigments. No mention of the presence of sterols in these plants is to be found in the literature and little is known of the carotenoid pigments. Willstätter and Page [1914] showed that the marine green alga *Ulva lactuca* contained only carotene and xanthophyll, and Kylin [1927] using qualitative capillary analytical methods for detection, but without actually isolating any pigments, stated that the lipochromes existent in the stated species were as shown in the following table:

	Carotene	Phyllorhodin	Xanthophyll	Phylloxanthin	Fucoxanthin
Higher plants	+	+	+	+	–
Chlorophyceae	+	+	+	+	–
Rhodophyceae	+	+	+	–	–
Phaeophyceae	+	–	+	+	+

The presumed pigments, phyllorhodin, phylloxanthin and even xanthophyll require re-identification in the light of our present knowledge of the plant lipochromes.

In our present study we have examined three freshwater Chlorophyceae, *viz.*: *Cladophora sauteri*, *Oedogonium* and *Nitella opaca*, and have taken *Rhodymenia palmata* as a typical member of the Rhodophyceae.

EXPERIMENTAL.

General procedure.

Cladophora sauteri (150 kg. wet weight) collected from Hickling Broad, Norfolk, was covered with methylated spirit (300 litres) and allowed to stand for 24 hours with intermittent agitation. The deep olive-green extract after concentration to one-third its volume was saponified at room temperature for 24 hours by the addition of 20 % aqueous KOH (2 litres). The whole was diluted with water, exhaustively extracted with ether, the ethereal solution washed with water and dried over sodium sulphate. In the case of *Rhodymenia palmata* (50 kg. wet weight) (collected from the north coast of Scotland), the plant was washed free from mucilages with 40 % acetone [see Heilbron and Phipers, 1935] and extracted with methylated spirit (50 litres), the alcoholic extract being saponified and the unsaponifiable material transferred to ether as described above.

The sterols were obtained from the non-saponifiable fractions by removing the solvent, dissolving the residues in the minimum quantity of boiling methyl alcohol and cooling to −75°.

The unsaponifiable fractions of *Nitella opaca* and *Oedogonium* were both obtained from the Torry Research Station, Aberdeen, the sterols being isolated by us by crystallisation from methyl alcohol as above.

STEROLS AND HENTRIACONTANE.

(*a*) Cladophora sauteri.

The pale yellow semi-crystalline mass was repeatedly crystallised from methyl alcohol giving (i) a small amount of a sparingly soluble waxy material which after several recrystallisations from ethyl acetate melted at 67–68° and was shown to be the paraffin hydrocarbon, hentriacontane, previously recognised as a constituent of the Phaeophyceae by Heilbron *et al.* [1934] and (ii) a more soluble portion, obviously sterolic, M.P. 80–120°. This latter was acetylated in the usual manner and a portion of the acetylated product (3 g.) in ether (30 ml.) treated with a 5 % solution of bromine in glacial acetic acid (40 ml.). After standing in the ice-chest for 24 hours the precipitated white powder, M.P. 130°, was directly debrominated by the method of Windaus and Hauth [1906], yielding after recrystallisation an acetate, M.P. 118–119°, which gave no depression in melting-point on admixture with an authentic sample of fucosteryl acetate. Hydrolysis of the acetate furnished fucosterol, M.P. 124°. After the removal of the fucosteryl acetate tetrabromide the mother-liquors were precipitated with methyl alcohol and the ether removed in a current of air. Crystallisation of the deposited material from methyl alcohol yielded a bromide, M.P. 120° (sitosteryl acetate dibromide melts at 120° [Anderson *et al.*, 1926]), debromination of which gave an acetate, M.P. 135°, $[\alpha]_D^{20°}$ −45·12° ($c=8$ in chloroform), which on hydrolysis furnished the free sterol, M.P. 136°. (Found: C, 84·1; H, 11·8 %. $C_{29}H_{50}O$ requires C, 84·05; H, 11·9 %.) The benzoate, prepared in pyridine with benzoyl chloride, melted at 143°.

The confused state of the sitosterol literature [compare Anderson *et al.*, 1926; Bonstedt, 1928] makes it difficult to assign a specific name to our sitosterol but from the appended table it would appear that it is a mixture of the *β*- and *γ*-forms.

	Sterol M.P.	Acetate M.P.	Benzoate M.P.	Rotation of acetate
α-Sitosterol	135–136°	127–128°	145°	−17·2°
β-Sitosterol	139–140	127–128	145	−39·0
γ-Sitosterol	142	143	—	−46·1
Algal sitosterol	136	135	143	−45·1

(*b*) Nitella opaca.

The crude mass was taken up in hot light petroleum, leaving a small amount (5 mg.) of an insoluble material, M.P. 280°, which was purified by crystallisation from aqueous pyridine, giving a micro-crystalline powder, M.P. 283–285°. (Found: C, 72·4; H, 10·3 %. $C_{35}H_{66}O_6$ requires C, 72·7; H, 10·5 %.) This compound responds to both the Liebermann-Burchard and the Salkowski tests, and this fact, taken in conjunction with the analytical data, indicates that it is a phytosterolin, a class of compound originally discovered by Power and Salway [1913] and more recently examined by Heyl and Larsen [1934].

The light petroleum solution was evaporated and the residue crystallised from methyl alcohol, giving a small amount of a sparingly soluble low-melting residue which on recrystallisation from ethyl acetate yielded hentriacontane,

M.P. 67–68°. The crude sterols obtained on concentration of the methyl alcoholic mother-liquors were separated by bromination, and fucosteryl acetate tetrabromide, M.P. 131–133°, and sitosteryl acetate dibromide, M.P. 120°, were thus obtained.

(c) Oedogonium.

Similar treatment of the crude material yielded a small amount (2 mg.) of the same phytosterolin, M.P. 283–285°, as obtained above; the sterol fraction on crystallisation from methyl alcohol yielded sitosterol, M.P. 135°; no trace of fucosterol could be found but hentriacontane was again detected.

(d) Rhodymenia palmata.

The crude sterols, M.P. 114–115°, on repeated crystallisation from methyl alcohol, furnished hentriacontane and fucosterol, M.P. 122–123°. Bromination of a portion of the crude sterol acetate gave only fucosteryl acetate tetrabromide, M.P. 130–132°.

CAROTENOID PIGMENTS.

The deep crimson methyl alcoholic solutions obtained after removal of the sterols in the manner described were subjected to the Kraus partition with 90 % methyl alcohol and light petroleum.

Cladophora sauteri.

(a) *The light petroleum phase.* Adsorption on activated alumina and development of the chromatogram with light petroleum gave three distinct orange-red bands. The uppermost band, on elution with methyl alcohol and subsequent crystallisation from the same solvent yielded a deep red solid, M.P. 175–177° (uncorr.), having absorption maxima in carbon disulphide at 505 and 473 $m\mu$ (see later).

Attempts to crystallise the concentrated methyl alcoholic eluate of the second zone were abortive owing to the presence of terpenes and sterols. We have now found that lipochromes can be completely freed from terpenes by adsorption on fuller's earth. Whereas the pigments are strongly adsorbed, prolonged washing of the developed chromatogram with benzene effectively removes all terpene. The eluted pigment solution, now only contaminated with sterol, was further purified by adsorption on calcium carbonate; in this case the pigment passed down the column, the sterol being retained in the upper section. The pigment obtained by concentration of the final methyl alcoholic eluate yielded, on crystallisation from the same solvent, lutein as a fine red powder, M.P. 187° (uncorr.), having absorption maxima in carbon disulphide at 512 and 479 $m\mu$.

The third band passed rapidly through the column and was collected in the filtrate. Removal of solvent gave a deep red odoriferous oil from which terpenes were removed by the process described above. Elution of the pigment and repeated crystallisation from methyl alcohol-benzene furnished β-carotene in small glistening coppery plates, M.P. 178–179° (uncorr.), having absorption maxima in carbon disulphide at 518, 483 and 450 $m\mu$.

(b) *The methyl alcoholic phase.* The solvent was removed and the residue dissolved in a mixture of benzene-light petroleum (1:3) and adsorbed on activated alumina, giving a very complex chromatogram consisting of four orange-red zones. Since the eluates of the zones were spectroscopically identical (maxima in carbon disulphide at 505, 474 and 446 $m\mu$) the complexity of the chromatogram is definitely due to sterol and terpene contaminants. Concentration of the eluates yielded red solids, M.P. 175–178° (uncorr.), which were combined with the pigment showing similar absorption maxima from the light petroleum chromatogram. Kuhn and Lederer [1932] have obtained a pigment showing the same absorption maxima from *Leontodon autumnalis*; by repeated chromatographic treatment on calcium carbonate these authors finally resolved it into taraxanthin and lutein. Adopting a similar procedure the above pigment, M.P. 175–178°, was resolved from benzene-light petroleum (1:3) by adsorption first on calcium carbonate and finally on activated alumina, taraxanthin and a further quantity of lutein being thus obtained. Crystallisation of the taraxanthin from methyl alcohol containing a trace of ether yielded glistening deep orange-red needles, M.P. 178–179° (uncorr.), having absorption maxima in carbon disulphide at 502, 472 and 442 mμ. Analytical values for the mixed pigment (C, 83·1; H, 9·8 %) indicate that the ratio of lutein to taraxanthin is approximately 7:3.

Nitella opaca.

(*a*) *Light petroleum phase.* The solution, after adsorption and development on calcium carbonate, showed two distinct red zones, the lower and more intense of which on elution and crystallisation from benzene-methyl alcohol furnished β-carotene, whilst the upper and smaller band was found to be spectroscopically identical with lutein.

(*b*) *Methyl alcoholic phase.* The deep red residue obtained on removal of solvent was dissolved in a mixture of light petroleum-benzene (2:1) and adsorbed on activated alumina. Development of the chromatogram gave two broad orange-red bands, the lower of which on elution and crystallisation from benzene-methyl alcohol furnished lutein in glistening deep red plates, M.P. 189–190° (uncorr.) having absorption maxima in carbon disulphide at 511, 479 and 445 mμ). The upper zone contained only a small amount of pigment, M.P. 175–176°, spectroscopically identical with taraxanthin.

Oedogonium.

(*a*) *Light petroleum phase.* The solution after adsorption on calcium carbonate gave a chromatogram showing three distinct bands, the upper of which, as shown by Kraus partition, consisted of traces of xanthophylls which had escaped partition. The second and main band furnished β-carotene, while the third, which was of a bright yellow colour, was spectroscopically characterised as α-carotene (absorption maxima in carbon disulphide at 510 and 478 mμ).

(*b*) *Methyl alcoholic phase.* The pigments were adsorbed on activated alumina as previously described, when two distinct bands were obtained, the upper of which yielded taraxanthin; the lower band contained a pigment, M.P. 175–177°, which after re-adsorption on calcium carbonate was resolved into lutein and taraxanthin.

Rhodymenia palmata.

(*a*) *Light petroleum phase.* The lipochromes, which were resolved in the usual manner, consisted of β-carotene and a trace of lutein.

(*b*) *Methyl alcoholic phase.* Chromatographic analysis yielded results identical with those obtained from the corresponding phase derived from *Cladophora sauteri*, the pigments thus being taraxanthin and lutein.

DISCUSSION.

A review of the relationship between algal species and their lipoid contents reveals many striking features as shown in Table I. It will be observed that each group of algae possesses characteristic lipochromes, the Chlorophyceae and

Table I.

			Lipochromes					Sterols	
		Hentriacontane	β-Carotene	α-Carotene	Lutein	Taraxanthin	Fucoxanthin	Sitosterol	Fucosterol
	Land plants	+	+	+	+	+	−	+	−
Chlorophyceae	*Oedogonium*	+	+	+	+	+	−	+	−
	Cladophora sauteri	+	+	−	+	+	−	+	+
	Nitella opaca	+	+	−	+	+	−	+	+
Rhodophyceae	*Rhodymenia palmata*	+	+	−	+	+	−	−	+
Phaeophyceae	*Pelvetia canaliculata**	+	+	−	−	−	+	−	+
	Fucus vesiculosus	+	+	−	−	−	+	−	+
	*Laminaria digitata**	+	+	−	−	−	+	−	+

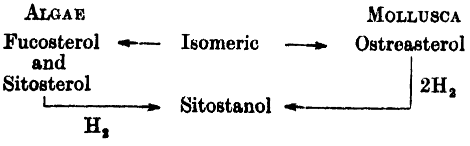
* Unpublished investigations.

Rhodophyceae containing taraxanthin and lutein in contrast to the Phaeophyceae which are distinguished by the presence of fucoxanthin [Heilbron and Phipers, 1935]. A gradual transition from the constituents of the normal land plants is observed through the species, the Phaeophyceae bearing little resemblance to the former.

According to Fritsch [1935] the botanical properties indicate that the Oedogoniales "are a sharply circumscribed group, showing but few points of affinity with the other Chlorophyceae". Our investigations provide strong proof of this statement, and indeed as far as the chemical composition of the lipoids is concerned, these plants can be considered as land plants. The normal Chlorophyceae contain both the phytosterol, sitosterol and the algal sterol, fucosterol. From the Rhodophyceae, sitosterol is absent, only fucosterol being observed; although this group does not contain the land plants' sterol, it still retains the typical phytoxanthophylls, *viz.* taraxanthin and lutein. In the Phaeophyceae even this last relationship with the land plants is severed, the characteristic pigment fucoxanthin being substituted.

Turning now to the results reported by Kylin [1927] and comparing them with our findings (see Table I) the identity of phyllorhodin with taraxanthin and of xanthophyll with lutein is evident. We are extremely doubtful as to the existence of a lipochrome, phylloxanthin, which Kylin states gives a green colour with 25 % hydrochloric acid, for we have repeatedly observed such a phenomenon with crude algal pigment solutions. Careful resolution, however, invariably yielded pigments giving no such colour on treatment with acid (see also preceding paper).

Reference has also been made [Heilbron *et al.*, 1934] to the interesting relationship existing between fucosterol and the isomeric sterol of the molluscs, ostreasterol (a dehydrositosterol) [Bergmann, 1934]. The evidence now brought to light that many species of algae also contain sitosterol further emphasises the very close biological relationship between these three sterols:



SUMMARY.

The unsaponifiable material from *Cladophora sauteri*, *Nitella opaca*, *Oedogonium* and *Rhodymenia palmata* has been examined. A clear-cut change in the character of the lipoids is observed when passing from one species to another.

(1) Whereas *Oedogonium* resembles the land plants in containing only sitosterol, *Cladophora sauteri* and *Nitella opaca* produce both the former sterol and fucosterol, the latter alone being present in the marine algae *Rhodymenia palmata* and *Fucus vesiculosus*.

(2) All algae contain small amounts of the paraffin hydrocarbon hentriacontane.

(3) A new phytosterolin, M.P. 283–285°, has been isolated from *Nitella opaca* and *Oedogonium*.

(4) The lipochrome pigments lutein, taraxanthin and β-carotene were isolated from all species and α-carotene was observed in *Oedogonium*. This presents a marked contrast to the Phaeophyceae (see preceding paper).

The evidence adduced shows that the Oedogoniales show a closer chemical relationship to the land plants than to the typical algae.

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REFERENCES.

Anderson, Nabenheimer, Shriner and Burr (1926). *J. Amer. Chem. Soc.* **48**, 2972, 2976, 2987.
Bergmann (1934). *J. Biol. Chem.* **104**, 317, 553.
Bonstedt (1928). *Z. physiol. Chem.* **176**, 274.
Fritsch (1935). The structure and reproduction of the Algae, **1**, 296. (University Press, Cambridge.)
Heilbron and Phipers (1935). *Biochem. J.* **29**, 1369.
—— —— and Wright (1934). *J. Chem. Soc.* 1572.
Heyl and Larsen (1934). *J. Amer. Chem. Soc.* **56**, 942.
Kuhn and Lederer (1932). *Z. physiol. Chem.* **213**, 188.
Kylin (1927). *Z. physiol. Chem.* **166**, 39.
Power and Salway (1913). *J. Chem. Soc.* **103**, 399.
Willstätter and Page (1914). *Liebig's Ann.* **404**, 237.
Windaus and Hauth (1906). *Ber. deutsch. chem. Ges.* **39**, 4380.

CLXVIII. THE ALGAE.

III. THE ISOLATION OF LACTOFLAVIN.

By ISIDOR MORRIS HEILBRON, EDRYD GWYLFA PARRY
and RICHARD FRANCIS PHIPERS.

From the Chemical Department, University of Manchester.

(*Received April 6th, 1935.*)

Whilst lactoflavin is widely distributed in both the animal [Kuhn *et al.*, 1934] and vegetable kingdoms [György *et al.*, 1934], it is still uncertain whether this flavin is the sole member of the group or whether there are several such compounds, *i.e.* hepatoflavin from horse liver [Stern, 1933], hepaflavin from malt [Karrer *et al.*, 1934], uroflavin from urine [Koschara, 1934] and renoflavin from ox kidney [Guha and Biswas, 1934].

In order to investigate the possible presence of lyochromes in the algae and to ascertain whether the flavin, if present, is identical with lactoflavin, we have examined the marine brown alga *Fucus vesiculosus* and the fresh water green alga *Cladophora sauteri* in detail.

During our investigations a communication appeared by Euler and Adler [1934] in which they claim to have detected the presence of flavins in several marine Phaeophyceae. As, however, these authors relied solely upon spectroscopic evidence, the question as to the identity still remained open.

EXPERIMENTAL.

Fucus vesiculosus.

The pale brown aqueous acetone washings from the fresh alga (50 kg. wet weight) [see Heilbron and Phipers, 1935] were very glutinous owing to large amounts of mucilage, thus rendering the usual procedure for the isolation of flavins by adsorption on fuller's earth impracticable. To overcome this difficulty we employed the methods described by Warburg and Christian [1933] whereby the photo-derivative lumilactoflavin is obtained. A portion of the extract (10 l.) was freed from acetone and made $N/2$ with respect to alkali by the addition of aqueous sodium hydroxide (380 ml. of $13N$). After irradiation, the lumiflavin was extracted from the acidified aqueous solution with chloroform. The bright yellow pigment, after purification by conversion into the sodium salt and finally by crystallisation from water, melted at 310° decomp. (uncorr.) and exhibited an absorption maximum in chloroform at $447 m\mu$. Warburg and Christian give for lumilactoflavin M.P. 320° (corr.) and an absorption maximum in chloroform at 445–$455 m\mu$. Kuhn, Rudy and Wagner-Jauregg [1933] give M.P. 328° (corr.) and report an absorption maximum in chloroform at $445 m\mu$.

Cladophora sauteri.

The methods described by Kuhn and Kaltschmitt [1935] for extraction of lactoflavin from hay were employed. The alga (50 kg. wet weight) was covered with hot dilute hydrochloric acid (50 l. at p_H 1·2) and allowed to stand with intermittent agitation for 12 hours. The flavin was removed from this aqueous extract by adsorption on fuller's earth and further purified by adsorption on lead sulphide and frankonite KL. The pigment was acetylated and chromatographically separated from impurities on a column of alumina. After elution, crystallisation of the pigment from aqueous methyl alcohol yielded red-brown crystals decomposing at 230° (uncorr.) with slight sintering at 180° (absorption maxima in water at 445 and $360 m\mu$). Kuhn and Wagner-Jauregg [1933] give for tetra-acetyl-lactoflavin M.P. 235° decomp. (corr.) and absorption maxima in water at 445 and $374–376 m\mu$.

SUMMARY.

Marine and fresh water algae as typified by *Fucus vesiculosus* and *Cladophora sauteri* both contain flavins. Lactoflavin has been obtained from the latter alga by direct adsorption from the aqueous extract, a process inapplicable to the former from which the photo-derivative lumilactoflavin was isolated.

We wish to thank Mr A. E. Gillam of this Department for the spectroscopic data, and the Department of Scientific and Industrial Research for a grant to further this research.

REFERENCES.

Euler and Adler (1934). *Z. physiol. Chem.* **228**, 12.
Guha and Biswas (1934). *Current Science*, **2**, 474.
Gyorgy, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 21.
Heilbron and Phipers (1935). *Biochem. J.* **29**, 1369.
Karrer, Salomon and Schopp (1934). *Helv. Chim. Acta*, **17**, 419.
Koschara (1934). *Ber. deutsch. chem. Ges.* **67**, 761.
Kuhn and Kaltschmitt (1935). *Ber. deutsch. chem. Ges.* **68**, 128.
—— Rudy and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1950.
—— and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1577.
—— —— and Kaltschmitt (1934). *Ber. deutsch. chem. Ges.* **67**, 1452.
Stern (1933). *Nature*, **132**, 784.
Warburg and Christian (1933). *Biochem. Z.* **257**, 492.

CLXIX. THE LIPOCHROMES OF SEA ANEMONES.

I. CAROTENOID PIGMENTS OF *ACTINIA EQUINA*, *ANEMONIA SULCATA*, *ACTINOLOBA DIANTHUS* AND *TEALIA FELINA*.

By ISIDOR MORRIS HEILBRON, HAROLD JACKSON
and RICHARD NORMAN JONES.

From the Department of Chemistry, University of Manchester.

(*Received April 6th, 1935.*)

Although during the past few years the structure of the plant lipochromes has been more or less completely elucidated, comparatively little is as yet known concerning the polyene pigments of marine animals. Indeed, up to the present, only one such pigment, astacene, which appears to be widely distributed in marine fauna, has been isolated and its structure determined [Kuhn and Lederer, 1933; Kuhn, Lederer and Deutsch, 1933; Karrer and Benz, 1934; Karrer and Loewe, 1934; Karrer *et al.*, 1935].

Euler *et al.* [1933] have described the occurrence of a pigment, salmenic acid, in the salmon whilst Euler and Hellström [1934] have isolated a further pigment, asterinic acid, from the starfish (*Asterias rubens*). Lönnberg [1931, 1, 2; 1932; 1933] has recorded spectroscopic data for the pigment extracts of numerous species of marine invertebrates. Various other pigments have been isolated by Lederer [1933; 1934, 1, 2] from different species of ascidia, mollusca and crustacea, the more important being pectenoxanthin, m.p. 182°, from the mollusc, *Pectin maximus*; glycymérine, m.p. 148–153°, from the scollop, *Pectunculus glycymeris*, and cynthiaxanthin, m.p. 188–190°, from the ascidian, *Halocynthia papillosa*. None of the above pigments has been examined in any great detail owing to the extreme difficulty of obtaining them in adequate quantity.

In the case of the pigments of various species of sea anemone, only one member has previously received attention. From *Actinia equina* Fabre and Lederer [1934] isolated a pigment, actinioerythrin, m.p. 85°, which they showed was a carotenoid ester. Prior to the appearance of these authors' paper we were ourselves engaged upon an examination of this pigment and, working on a larger scale, have obtained from it a hydrolysis product, m.p. 191–192°, which is, so far as we know, unique among carotenoids both as regards colour and absorption spectrum. Whereas actinioerythrin has absorption maxima in carbon disulphide at 574, 538 and 497$m\mu$ the new pigment, for which we propose the name "*violerythrin*", shows maxima in the same solvent at 625, 576 and 540$m\mu$.

In addition to the above, three other species, *Actinoloba dianthus*, *Tealia felina* and *Anemonia sulcata*, have so far been examined. Like *Actinia equina*, both *Actinoloba dianthus* and *Tealia felina* contain, apart from carotene, only esterified carotenoids. The red pigment of *Actinoloba dianthus* is readily hydrolysed, yielding an acid, m.p. 195–196°, both acid and ester showing a single absorption maximum in carbon disulphide near 495$m\mu$. From *Tealia felina* two pigments have been isolated; of these one is possibly identical with actinioerythrin whilst the second on hydrolysis yields an acid, m.p. 205–208°, with a single absorption maximum near 500 mμ in carbon disulphide. *Anemonia sulcata* differs from the other three species in containing no esterified pigments. The main lipochrome, *sulcatoxanthin*, for which the formula $C_{40}H_{52}O_8$ is suggested by analytical data, is characterised by three absorption maxima in carbon disulphide at 516, 482 and 450 mμ.

The present communication deals with the method of isolation of the pigments and certain preliminary observations as to their nature.

EXPERIMENTAL.

1. Actinia equina.

Isolation of actinioerythrin. The anemones were minced and repeatedly extracted with a mixture of ether and acetone (1:1) at room temperature. The combined extracts were concentrated to small bulk and diluted with water and the pigment was transferred to light petroleum. Phosphatides and sterols were removed by precipitation from the concentrated light petroleum solution with acetone, followed by further concentration and freezing to − 10°. The pigment was again transferred to light petroleum and adsorbed on alumina (Merck standardised Brockmann diluted with an equal volume of inactive alumina); the chromatogram was developed with benzene; the actinioerythrin formed an intense violet-black zone on the upper half of the column, whilst a yellow pigment, spectroscopically identified as carotene, washed rapidly through. The ester was eluted with light petroleum-methyl alcohol and readsorbed from light petroleum on calcium carbonate, whereby remaining traces of sterol, terpene material and a further yellow pigment (absorption maxima at 502, 472 and 442 mμ in carbon disulphide and probably a taraxanthin ester) were removed. After elution and removal of solvent, the crude actinioerythrin was crystallised from absolute alcohol giving microscopic red-black needles, M.P. 83° (uncorr.) (Fabre and Lederer [1934] give M.P. 85°). In agreement with these authors we find that whereas in carbon disulphide the pigment is characterised by three absorption maxima at 574, 538 and 497 mμ, in alcohol only one band with head at 511 mμ is present. On partition between light petroleum and 90 % methyl alcohol actinioerythrin is wholly epiphasic (the yield of crystalline pigment from 500 anemones is approximately 30 mg.).

Isolation of violerythrin. Fabre and Lederer [1934], working with a minute quantity of the ester, noted its ready hydrolysis but state that the resultant "acid" decomposed on liberation. We have found, however, that under carefully controlled conditions of saponification a crystalline product can be isolated. As a result of numerous experiments the following method has been found the most satisfactory. A solution of actinioerythrin (5 mg.) in light petroleum was saponified by shaking at room temperature with methyl alcoholic sodium hydroxide (20 ml. of 2·5 %) until the pigment was completely transferred to the alcohol phase (approximately 1 hour). The latter was diluted with water and rendered faintly acid with 5 % acetic acid, the end-point being readily observed by a colour change from cherry-red to orange. The pigment was extracted with ether and the washed solution dried over anhydrous sodium sulphate. After removal of solvent the crude solid was recrystallised from aqueous pyridine, when violerythrin (1 mg.) separated in deep violet microcrystals, M.P. 191–192° (absorption maxima at 625, 576 and 540 mμ in carbon disulphide). The pigment exhibits a remarkable range of colours in various solvents; in benzene it is deep blue, in pyridine and acetone bright blue, in ether and alcohol violet-red and in carbon disulphide purple.

From the above it would be anticipated that violerythrin is itself acidic in nature but this does not appear to be the case since dilute alkali is without action upon it. Probably the initially formed "acid" rearranges from an enolic to a stable ketonic form; more detailed study of the hydrolytic process is being carried out.

2. Anemonia sulcata.

500 animals were finely minced and extracted with ether-acetone (1:1). Whereas the initial extracts were of a deep reddish yellow colour, the later ones were typically chlorophyll-like in character. The combined extracts were concentrated, diluted with water and extracted with light petroleum and the resulting solution was partitioned with 65 % methyl alcohol. The residual petroleum phase was found to contain, in addition to carotene and chlorophyll, a small quantity of a xanthophyll pigment (absorption maxima at 478 and 450$m\mu$ in carbon disulphide) which passed readily into 90 % methyl alcohol.

The sulcatoxanthin contained in the 65 % methyl alcohol was transferred to benzene and adsorbed on activated alumina. The chromatogram was developed by washing with benzene-ether (4:1), the pigment being held as a bright red zone near the head of the column. After elution and removal of solvent, the residue was dissolved in carbon disulphide and readsorbed on calcium carbonate. On addition of light petroleum to the concentrated ether eluate the sulcatoxanthin separated as a flocculent deep scarlet precipitate (50 mg.).

Properties of sulcatoxanthin. The pigment is insoluble in light petroleum, sparingly soluble in carbon disulphide and readily soluble in benzene and alcohol. It possesses no sharp melting-point but suddenly shrinks at 110°, softens at 125° and is completely melted at 130°. In carbon disulphide it shows three absorption bands with maxima at about 516, 482 and (450)$m\mu$. It is strongly adsorbed on sugar from carbon disulphide as a uniform rose-coloured zone (further evidence of its homogeneity) and this, together with its ready partition into 65 % methyl alcohol, is clearly indicative of a high state of oxidation. Treatment with alkali, even under mild conditions, causes complete destruction of the pigment, whilst with concentrated sulphuric acid a blue colour is produced. (Found: C, 72·4, 72·4; H, 8·1, 7·9 %. $C_{40}H_{52}O_8$ requires C, 72·7; H, 7·9 %.)

Chlorophyll-a from Anemonia sulcata. The presence of a chlorophyll-like substance in this species was first observed by MacMunn [1885] and confirmed by Elmhirst and Sharpe [1920], who attributed its occurrence to the presence of symbiotic algae in the tentacles. We have identified this pigment as chlorophyll-a in the following manner. The tentacles were removed from a dozen freshly killed animals and the total pigments transferred to light petroleum (B.P. 60–80°). The sulcatoxanthin was removed with 90 % methyl alcohol and the residual emerald-green solution examined spectroscopically and found to be identical with pure chlorophyll-a examined in the same solvent (absorption maxima at 657, 612, 571, 532, 504, 494, general abs. from 465$m\mu$).

3. Actinoloba dianthus.

The pigment from 50 anemones was obtained in light petroleum solution in the usual manner. Partition with 90 % methyl alcohol indicated the presence of epiphasic carotenoids only. By adsorption of the light petroleum solution on calcium hydroxide one pigment was retained as a reddish brown band whilst a second passed through the column (absorption maxima at 501 and 471$m\mu$ in carbon disulphide). The adsorbed pigment was transferred to light petroleum and shaken with calcium carbonate by which it was readily held. From this a deep red, low-melting solid was obtained which could not be crystallised. On saponification with alcoholic sodium hydroxide (10 ml. of 5 %) at room temperature the ester was rapidly hydrolysed, giving on dilution an insoluble red sodium salt. The free acid crystallises from aqueous pyridine in deep violet-red prisms, M.P. 195–196° (2 mg.), showing a single absorption maximum in carbon disulphide at 495$m\mu$.

4. Tealia felina.

The degree of pigmentation of this species of anemone varies considerably but on the whole is small. Two varieties were examined, a small shore form and a larger deep water animal. The pigments were obtained in light petroleum and a partition experiment with 90 % methyl alcohol showed the absence of hypophasic carotenoids. The chromatogram, developed with benzene on activated alumina, exhibited a violet-red zone, whilst a yellow pigment (carotene) washed through the column. Re-adsorption on calcium carbonate furnished three zones, coloured violet, red and yellow respectively, the last being present in only minute amount. The violet zone yielded, after crystallisation from absolute alcohol, a violet-black wax, M.P. 65–73°, with absorption maxima in carbon disulphide at 569, 530 and 497$m\mu$. This pigment shows a close resemblance to actinioerythrin, but definite identification can only be established when larger quantities are available.

From the red zone an orange-red low-melting ester was obtained (absorption maximum at 500$m\mu$ in carbon disulphide), hydrolysing to give on dilution an insoluble red sodium salt. The free pigment, obtained in the usual manner, separated from aqueous pyridine in black feathery needles, M.P. 205–208° (1 mg.), having the same absorption spectrum as the parent ester.

SUMMARY.

1. A preliminary investigation into the carotenoid pigments of four species of sea anemone has been made.

2. Actinioerythrin (M.P. 85°), the principal pigment of *Actinia equina*, has been isolated and hydrolysed to a non-acidic pigment, *violerythrin*, M.P. 191–192°.

3. The pigment of *Anemonia sulcata* consists mainly of a new lipochrome, *sulcatoxanthin*, probably $C_{40}H_{52}O_8$. The presence of chlorophyll-a in the tentacles has been demonstrated.

4. From the species *Actinoloba dianthus* a red acidic pigment, M.P. 195–197°, has been isolated.

5. Two lipochrome esters, one possibly identical with actinioerythrin, have been isolated from *Tealia felina*.

6. Spectroscopic data concerning the above and other polyenes present in these species have been obtained.

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REFERENCES.

Elmhirst and Sharpe (1920). *Biochem. J.* **14**, 48.
Euler and Hellström (1934). *Z. physiol. Chem.* **223**, 89.
—— —— and Malmberg (1933). *Svensk. Kem. Tidskr.* **45**, 151.
Fabre and Lederer (1934). *Bull. Soc. Chim. Biol.* **16**, 105.
Karrer and Benz (1934). *Helv. Chim. Acta,* **17**, 412.
—— and Loewe (1934). *Helv. Chim. Acta,* **17**, 745.
—— —— and Hübner (1935). *Helv. Chim. Acta,* **18**, 96.
Kuhn and Lederer (1933). *Ber. deutsch. chem. Ges.* **66**, 488.
—— —— and Deutsch (1933). *Z. physiol. Chem.* **220**, 229.
Lederer (1933). *Compt. Rend. Soc. Biol.* **116**, 150.
—— (1934, 1). *Compt. Rend. Soc. Biol.* **117**, 411.
—— (1934, 2). *Compt. Rend. Soc. Biol.* **117**, 1086.
Lönnberg (1931, 1). *Ark. Zoo.* (Sweden), A **22**, 14.
—— (1931, 2). *Ark. Zoo.* (Sweden), A **23**, 15.
—— (1932). *Ark. Zoo.* (Sweden), A **25**, 1.
—— (1933). *Ark. Zoo.* (Sweden), A **26**, 7.
MacMunn (1885). *Phil. Trans. Roy. Soc. Lond.* **2**, 641.

CLXX. THE ASSIMILATION OF INORGANIC NITROGENOUS SALTS, INCLUDING SODIUM NITRITE, BY THE GRASS PLANT.

BY WILLIAM GEORGE ELFORD EGGLETON.

From the Imperial Chemical Industries, Agricultural Research Station, Jealotts Hill, Berks.

(*Received April 16th, 1935.*)

THE primary object of this investigation was to obtain information regarding the effect of applying sodium nitrite to grassland and to determine whether the nitrite accumulated in sufficient quantity in the herbage to cause metabolic disturbance. For comparative purposes, however, three other treatments were included: ammonium sulphate, sodium nitrate and potassium sulphate. By sampling and analysing the herbage at intervals after the application of the salts to the soil, it was hoped to follow to some extent the course of the elaboration of protein-N from the various forms of inorganic nitrogen assimilated by the plant. For this purpose the amide-N, pepsin-HCl-soluble N, true protein-N and total-N were determined as well as ammonia-N, nitrate-N and nitrite-N. It had been hoped to obtain a measure of the total amount of dry matter at each sampling, so that the nitrogen partition could be considered in terms of absolute weight per unit area, but unfortunately other considerations prevented this being done on a sufficiently accurate basis. Consequently the amounts of nitrogen present in various forms in the herbage are of necessity expressed as parts per 100 of dry matter, and this must be borne in mind when making comparisons. The course of the nitrogen transformations in the soil was followed to some extent by analyses of samples of soil taken from the various plots at time of grass sampling, the ammonia-N, nitrate-N and nitrite-N being determined in the soil extract.

EXPERIMENTAL.

The experimental plot, a five by five latin square, was situated in Drownboy Field, at this Station. The various salts were applied in solution from a small watering can fitted with a fine rose, in amounts corresponding to an application of ammonium sulphate at the rate of 3 cwt. per acre; the potash was applied at the rate of 2 cwt. per acre. The foliage was well watered afterwards to ensure that no salts adhered to the grass, the control plots receiving the same total quantity of water, 0·25 in., as the treated plots.

The soil was sampled to a depth of 3 inches with a soil sampling tube of 1 inch diameter, and the grass by plucking the required quantity from random positions over the five replicates.

So far as the analyses of the soil nitrogen are concerned, the methods used were substantially the same as those already described by the author [1934].

The "true protein"-N in the dried and finely ground grass was determined by Stützer's method, which involves precipitation of the proteins by a suspension of cupric hydroxide in glycerol and water and subsequent determination of the nitrogen content of the washed precipitate.

The amide-N was determined by the difference in ammonia-N content of a known weight of dried and ground grass before and after boiling with HCl (5 %) to convert amides into ammonium chloride.

The pepsin-HCl-soluble N, which may be taken as a good comparative measure of the digestibility of the herbage, was determined by comparing the total nitrogen content of the original dried and ground grass with that of the residue left after digestion with pepsin-HCl. Since the figure obtained includes inorganic nitrogen if present, the necessary corrections were subsequently applied.

The ammonia-N content of the dried and ground grass was determined by distillation with magnesia.

Estimation of nitrite-N in grass. Immediately after sampling, 10 g. of the wet grass, the dry matter content of which was subsequently determined, were macerated in a stone mortar with 50 g. of sand previously extracted with HCl and ignited. The grass was effectively disintegrated only when the very minimum of water was added. After maceration, the remainder of 100 ml. water was added, and the whole filtered on a Büchner funnel. An aliquot (50 ml.) of this filtrate was immediately clarified by the addition, in order, of 2 ml. of *N* $CuSO_4$, 0·5 g. $MgCO_3$ and 0·5–1·0 g. $Ca(OH)_2$. After filtration, the nitrite was determined colorimetrically on an aliquot by the Griess-Ilosva method, the whole operation not taking more than half an hour.

Estimation of nitrate-N in grass (*due to Mr F. L. Ashton*[1]). The method consisted of boiling the finely ground grass with water and determining the nitrate content of an aliquot part of the clarified extract by the phenoldisulphonic acid method. Since the accuracy of the method depends entirely on the efficiency of the clarification, both as regards removal of organic material and the non-removal of nitrate, a brief description of the method used to clarify the water extract is necessary. The bulk of the organic matter present was precipitated by the addition of a solution of basic lead acetate, and, after filtering and washing, the cold filtrate was shaken with a small quantity of "norite". The excess lead and any chlorides present were then precipitated by the addition of $(NH_4)_2SO_4$ and Ag_2SO_4 and the combined precipitates, including the norite, filtered off. The nitrate determination was then conducted colorimetrically on an aliquot of the filtrate after taking to dryness. In control experiments satisfactory recoveries of known amounts of added nitrate were obtained.

Nitrogen partition in the soil.

In Table I are set out the amounts of ammonia-N, nitrate-N and nitrite-N found in the soil on the various sampling dates.

The nitrogen was added at the rate of 69·5 lb. per acre which, since the weight of 1 acre of dry soil from Drownboy Field to a depth of 3 in. is $0 \cdot 9 \times 10^6$ lb., corresponds to 77 p.p.m. of dry soil. It will be seen that the figures for ammonium sulphate, sodium nitrate and sodium nitrite, given in Table I, fall short of this amount by 42, 49 and 54 p.p.m. respectively. Whilst, as is shown by Table III, some of this deficiency is undoubtedly due to assimilation by the grass during the interval between applying the salts and sampling the soil, by far the greater part must have arisen from inefficient separation of soil and root by the sieving process, particularly as regards the surface layer, where the added salts were mainly located. It is consistent with this that the amounts recovered were materially higher on the following day after a night's rain, when presumably the salts were washed down into layers where the smaller amount of organic débris

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[1] A detailed account of this method, including several modifications subsequently introduced, is now in the press.

Table I. *Inorganic nitrogen partition in the soil following the addition of various salts.*

p.p.m. dry soil.

Days commencing 1. v. 33 ...	1	2	3	9	24
Ammonia-N:					
Control	6·7	8·2	7·6	11·1	6·8
$(NH_4)_2SO_4$	5·6	34·9	43·4	14·8	10·0
$NaNO_3$	6·6	9·9	8·7	12·1	10·1
$NaNO_2$	7·0	8·7	13·7	14·9	9·4
K_2SO_4	6·3	6·5	13·3	5·9	9·8
Nitrate-N:					
Control	(12·7)	4·1	2·2	1·4	0·3
$(NH_4)_2SO_4$	2·8	1·7	0·5	2·5	1·0
$NaNO_3$	3·1	27·7	(37·2)	7·6	0·6
$NaNO_2$	2·1	0·1	1·4	2·7	0·6
K_2SO_4	3·5	1·0	12·7	2·1	0·6
(Some doubt attaches to the figures in brackets.)					
Nitrite-N:					
Control	<0·1	0·04	0·06	0·07	Traces
$(NH_4)_2SO_4$	<0·1	0·07	0·17	0·09	Traces
$NaNO_3$	<0·1	0·22	0·08	0·09	Traces
$NaNO_2$	<0·1	22·8	27·0	0·32	Traces
K_2SO_4	<0·1	0·06	0·25	0·06	Traces

permitted a more efficient separation of the soil. The deficiencies on this occasion were less; 34, 40 and 50 p.p.m. respectively. It is apparent from this that if the assimilation of nitrogenous salts were to be followed in any detail from the soil side, adequate provision would have to be made to ensure a much more effective separation of soil from root than that provided by the sieving method now employed. It might even be advisable to consider soil and root together by drying the soil sample *in vacuo*, sieving and re-incorporating the organic matter thus separated after grinding in a suitable mill.

However, in spite of the complication mentioned above, several points are abundantly clear. Firstly, only a very small fraction of the nitrogen originally added remained in the soil in the form of ammonia, nitrate or nitrite 8 days after addition. So far as can be judged from the above figures, ammonium sulphate has disappeared least quickly and sodium nitrite most quickly, with sodium nitrate intermediate. So much is in accord with accepted opinion. The disappearance of the nitrite, however, does not appear to have been accompanied by an increase in nitrate-N in the soil. Moreover, there was no corresponding accumulation of nitrite-N or nitrate-N in the herbage. For example, on the 24th day there were still 580 p.p.m. of nitrate-N in the grass which had been treated with sodium nitrate, but the grass which had been treated with sodium nitrite had by this time no more nitrate-N than it had at the beginning of the experiment, *viz.* 10 p.p.m. This rather gives the impression that the nitrite has been dealt with either in the soil or in the plant much more quickly than the nitrate and, moreover, in a way which presumably has not entailed its conversion into nitrate, in which case it should have been detected in this form either in the grass or in the soil. This might be explained by assuming an enhanced rate of synthesis of protein, but the fact that the amount of pepsin-HCl-soluble N, which fraction includes amino-acids, was lowered in the nitrite-treated grass (see Tables V and VII), lends colour to the view that a proportion of the nitrite had reacted with amino-compounds present in the grass with the resulting formation of free nitrogen.

Another feature of Table I is that the amount of ammonia-N in the soil was increased following the application not only of ammonium sulphate (which is, of course, expected), but also of sodium nitrate and, to a greater extent, of sodium nitrite. This increase in ammonia-N, which has been observed in the case of sodium nitrate on other occasions, indicates that some of the added nitrate and nitrite had undergone a process of reduction.

Finally, it may be noted that a slight increase in nitrite-N took place immediately following the application of sodium nitrate, although the added sodium nitrate was free from nitrite.

Nitrogen partition in the herbage.

All the nitrogenous salts had the effect of increasing the proportion of total nitrogen in the dry matter of the herbage, the greatest increase occurring with

Table II. *Increases in the percentage of total nitrogen in the dry matter of spring grass treated with various salts.*

Fertilisers added 2 hours before sampling on 2nd day.[*]

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	0·27	0·59	0·98	0·33
$NaNO_3$	—	0·27	0·52	1·15	0·46
$NaNO_2$	—	0·27	0·32	0·32	-0·07
K_2SO_4	—	0·24	0·69	0·37	0·19

(Mean initial percentage N in control = 3·93 ±0·12.[*])

[*] The estimate of error has been determined in all cases from the six values obtained from the five parallel observations on the first day together with that for the control on the second day.

Table III. *Inorganic nitrogen partition in the dry matter of spring grass before and after application of various salts.*

Application made 2 hours before sampling on 2nd day. Results in p.p.m.

Days commencing 1. v. 33 ...	1	2	3	9	24
Ammonia-N:					
Control	430	380	450	500	390
$(NH_4)_2SO_4$	390	920	640	830	320
$NaNO_3$	380	600	530	630	390
$NaNO_2$	420	490	420	420	250
K_2SO_4	380	500	480	480	320
		(S.E. = ±23.)			
Nitrate-N:					
Control	<10	<10	<10	<10	<10
$(NH_4)_2SO_4$	<10	<10	<10	510	290
$NaNO_3$	<10	880	560	1250	580
$NaNO_2$	<10	60	20	310	<10
K_2SO_4	<10	<10	<10	<10	<10
Nitrite-N:	(Accuracy approx. 10 p.p.m.)				
Control	0·25	0·7	f. tr.	—	—
$(NH_4)_2SO_4$	0·25	1·1	f. tr.	f. tr.	—
$NaNO_3$	0·25	4·3	f. tr.	f. tr.	—
$NaNO_2$	0·25	44·0	tr.	—	—
K_2SO_4	0·25	0·7	f. tr.	f. tr.	—
		(S.E. < ±0·1 p.p.m.)			
9 a.m. soil temperature (° F.) at 4 in. (10 cm.)	51·0	51·0	50·0	—	—
Sunshine (hours)	4·3	0·0	2·4	—	—

sodium nitrate and the least with sodium nitrite. Potassium sulphate also increased the total nitrogen content. The results are shown in Table II as increases relative to the control.

Ammonium sulphate, sodium nitrate, potassium sulphate and sodium nitrite all increased the proportion of ammonia-N in the dry matter, in order of descending magnitude, the maximum increase occurring shortly after application to the soil (see Table III). The increase in ammonia-N content of the herbage following application of potassium sulphate may be explained in terms of base exchange, the potash liberating ammonia from the soil colloid complex.

The proportion of nitrate-N was very markedly increased soon after addition of the sodium nitrate. Ammonium sulphate and sodium nitrite in the order mentioned also increased the nitrate content, but the increases were not immediately apparent. No change in nitrate content followed the application of the potassium sulphate.

The proportion of nitrite-N showed immediate increases in all cases where a nitrogenous salt was added, the increase being greatest for sodium nitrite and least for ammonium sulphate. Potash was without effect. Only faint traces of nitrite-N were detected in any treatment 24 hours after application, and none was detected subsequently. So far as the eye could judge, the grass suffered no harm from the application of sodium nitrite at the rate of 3 cwt. per acre.

Table IV. *Differences in percentage of amide-N in the dry matter of spring grass treated with various salts.*

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	−0·030	+0·033	+0·075	−0·039
$NaNO_3$	—	−0·041	+0·029	+0·052	−0·033
$NaNO_2$	—	−0·012	+0·044	+0·035	−0·043
K_2SO_4	—	−0·017	+0·034	−0·003	−0·012

(Mean value for control on 1st day = 0·109 % ± 0·013.)

The proportion of amide-N was increased after an initial drop, due to the application of other forms of nitrogen and reached a maximum about 8 days after application. At the end of the experiment the proportion of amide-N on all the treated plots was substantially lower than on the control.

Table V. *Increases in % pepsin-HCl-soluble N (less inorganic N) in the dry matter of spring grass, following treatment with various fertilisers.*

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	0·17	0·50	0·63	0·24
$NaNO_3$	—	0·12	0·45	0·88	0·41
$NaNO_2$	—	0·16	0·23	0·25	−0·03
K_2SO_4	—	0·17	0·71	0·37	0·13

(Mean value for control on 1st day = 2·81 % ± 0·08.)

The proportion of pepsin-HCl-soluble N was also increased, sodium nitrate showing the greatest and most permanent increase, followed in order by ammonium sulphate and potassium sulphate. The pepsin-HCl-soluble N was only slightly increased by the sodium nitrite treatment and was actually slightly less than that of the control at the end of the experiment.

A rise in the proportion of "true protein"-N occurred in all cases on the day after application; this increase was maintained with ammonium sulphate and sodium nitrate until the end of the experiment, but with sodium nitrite the proportion was substantially the same as in the control by the 9th day and was

Table VI. *Increases in °/₀ "true protein"-N in the dry matter of spring grass following treatment with various fertilisers.*

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	0·07	0·33	0·22	0·26
$NaNO_3$	—	0·13	0·36	0·32	0·26
$NaNO_2$	—	0·19	0·30	0·06	−0·25
K_2SO_4	—	0·02	0·44	−0·16	0·02

(Mean value for control on 1st day = 3·23 % ±0·08.)

markedly lower on the 24th day. Potassium sulphate increased the proportion of "true protein"-N on the day after application, but the increase did not persist.

It is of interest to consider the sum of the following forms of nitrogen, which according to the methods of analysis employed are mutually exclusive, in relation to the total amount of nitrogen found to be present by separate Kjeldahl determination.

Table VII. *Amount of nitrogen in spring grass in the form of "true protein", amide, NH_3, NO_3 and NO_2, expressed as °/₀ of the total N present.*

Days commencing 1. v. 33 ...	1	2	3	9	24
Control	88·6	89·3	88·8	98·5	101·6
$(NH_4)_2SO_4$	88·9	86·0	85·1	85·2	102·4
$NaNO_3$	88·1	87·9	88·3	84·4	98·3
$NaNO_2$	88·2	87·8	90·0	92·8	96·0
K_2SO_4	92·9	88·4	91·1	88·6	104·7

At the commencement of the experiment all four samples agree in showing that between 7 and 12 % of the nitrogen known to be present remains undetermined. On the 24th day of the experiment this undetermined fraction, which, from the nature of the analysis, consists of fairly simple amino-acids, peptides, and other simple soluble organic nitrogen compounds other than amides, not precipitable with Stützer's reagent, has decreased to negligible proportions in all cases; indeed, on the control grass this state of affairs has been reached by the 9th day. It would appear, therefore, that by the beginning of May the rate of protein synthesis is such that the margin of partially elaborated nitrogen compounds is extremely small, and that this condition may not be reached until some weeks later when inorganic nitrogenous salts are added to the soil.

The presence of nitrite-N in the expressed juice of spring grass.

Schimper [1888] showed that nitrites are always present in the living leaf when the plant is in the dark, and that they disappear when the leaf is exposed to light. With regard to its occurrence in the plant under illuminated conditions, Rimington and Quin [1933] have found traces in the fresh juice of certain members of the genus *Tribulus*. It has also been detected in the stem of grape vines, in the leaves of carrot and in the leaves of soya bean in the early stages of growth, but so far as can be ascertained, its occurrence normally in spring grass has not been recorded. The amounts found by the author were always small (< 1 p.p.m. of dry matter).

In the experiment described above, the amount of nitrite in the expressed juice was increased within 2 hours of application to the soil, not only on the plots treated with sodium nitrite and sodium nitrate, but also on those treated with ammonium sulphate, a fact which seems to indicate that some at least of the ammonium sulphate was oxidised in the leaf. (There was no increase of nitrate in the leaf until a few days later.) Attention has been already drawn to the rapid assimilation of nitrogenous salts by Eckerson [1924], who observed nitrate in the leaves of tomato 24 hours after applying calcium nitrate to the roots—she also observed nitrite 36 hours after treatment.

Mevius and Dikussar [1930] showed that in neutral or alkaline solution *Zea Mays* could utilise nitrites without metabolic disturbances. They found that the optimum nitrite concentration was about 50 p.p.m. in a neutral solution, and that at greater concentrations protein synthesis was retarded with simultaneous accumulation of amides and ammonia but not nitrite. In the experiment recorded here no evidence was obtained of accumulation of the forms of nitrogen mentioned. During the first 9 days after application the nitrite materially increased the percentage of true protein-N and pepsin-HCl-soluble N, as did the other fertilisers tested but to a smaller extent, but by the 24th day after application, the percentages of total-N and pepsin-HCl-soluble N, true protein-N, amide-N and ammonia-N were found to be depressed below the corresponding values in the untreated grass, although the applied nitrite had already disappeared from the soil and the herbage by the 9th day. The highest concentration of soil nitrite-N recorded in this experiment was 27 p.p.m., corresponding to 190 p.p.m. of soil solution, and the p_H of the soil was 6·8.

The temporary accumulation of nitrite in unmanured grass is undoubtedly conditioned by rapid assimilation of nutrients and by low intensity of solar radiation, a state of affairs which may be presumed likely to occur only in early spring or late autumn (or in summer under heavy shade). It appears likely, therefore, that it is a contributory factor in the so-called "grass tetany" of cattle which is sometimes observed at these times and is usually associated with high inorganic nitrogen content of the herbage.

In this connection it is of interest to record an observation made at Jealotts Hill. On May 9th, 1933 it was noticed that stock had broken out of a field (A) which had been treated with nitrochalk a few days previously. They were found grazing contentedly in the adjoining field (B) which had not been so treated. To the eye, no difference in the herbage of the two fields was apparent, but in view of the possibility that the stock had detected some disagreeable quality in the herbage, samples were taken from both fields and examined for nitrite by the above method, with the following results.

Table VIII.

	Nitrite-N		Potassium nitrite
	p.p.m. wet grass	p.p.m. dry matter	g./cow/day*
Field A	1·2	7·0	0·6
Field B	0·4	2·0	0·19

* Assuming a daily consumption of 30 lb. dry matter per cow per day.

Thus it will be seen that there was at least three times as much nitrite present in the grass which the stock found unpleasant as in the grass which they found palatable.

Unfortunately nitrite was the only nitrogenous constituent looked for on this occasion, so that there still remains the probability that nitrate was also present in the treated grass, although this is known to be considerably less toxic than nitrite. Nevertheless, apart from the certain amount of circumstantial evidence concerning the part played by nitrite in "grass tetany", the figures demonstrate that nitrite occurs naturally in grass in the spring, and that the amount may increase after treatment with a nitrogenous manure.

The significance of nitrite-N in protein synthesis.

Berthelot and Gaudechon [1911] demonstrated nearly twenty-five years ago that nitrite was formed when solutions of ammonium salts were exposed to ultraviolet radiation, and more recently Dhar *et al.* [1933] and Corbet [1934] have confirmed that nitrite formation can be effected by ultraviolet light in the absence of micro-organisms. It would appear likely that the same reaction occurs in the grass leaves under the action of sunlight. The appearance of nitrite after the application of sulphate of ammonia certainly supports this view and raises the question whether the ammonia taken up as such all goes through the nitrite stage or whether part is directly synthesised into amino-compounds and only a fraction is converted into nitrite. In any case, the simultaneous occurrence of amino-compounds and nitrites may constitute a fruitful source of loss of nitrogen in the elementary form, the reaction forming what is probably a natural safeguard against undue accumulation of nitrite.

It might be mentioned in passing that the much vexed question whether ammonia-N or nitrate-N is more readily utilised by the grass plant has still to be answered, and although there is a mass of circumstantial evidence based on the observed ready uptake of applied ammonia by the plant under certain conditions, no conclusive evidence has yet been brought forward to show that nitrogen is freely taken up by grass in the ammoniacal form *in natura.* It is true that plants grown *in vitro* and supplied with ammonium salts as a sole source of nitrogen definitely do assimilate ammonium salts as such, but the conditions are far from natural. Even the entirely practical conditions of manuring in the field with ammonium sulphate, when again ammonia can be shown to be taken up, as in the above experiment, can hardly be described as normal to the plant Nevertheless, the point to be stressed is that even when the plant is faced with the necessity of dealing with its nitrogen in the ammoniacal form, a mechanism certainly exists whereby a part, if not all, can be oxidised to nitrite, thereafter to be dealt with possibly as suggested by Baly *et al.* [1922].

Work has been in progress in this laboratory on the photosynthesis of amino-acids from solutions of nitrite and sugars, and definite evidence of a reaction has been obtained, and recently also Dhar and Mukherjee [1934] have been successful in synthesising amino-acids by exposing solutions of glycol or glucose and nitrates to sunlight in the presence of titania as a catalyst. The reaction obviously proceeds through the nitrite stage. There would thus appear to be substantial grounds for believing that the photochemical formation of nitrite from nitrate and ammonia is of primary and fundamental importance in the synthesis of protein in plant tissue.

SUMMARY.

An attempt has been made to follow the transformation of various forms of inorganic nitrogen applied to the soil into elaborated organic nitrogen in the grass plant.

Under the conditions of the experiment, applied inorganic nitrogen was detected in substantial amounts in the herbage only 2 hours after application to the soil.

In early spring, synthetic processes in the leaf are slow enough to permit the accumulation of simple inorganic and organic compounds. This margin of partially elaborated nitrogen rapidly disappears, but at a diminished rate, when inorganic nitrogenous salts are applied to the soil.

Nitrite occurs naturally in spring grass, and the amount is increased when the grass is treated with ammonium sulphate or sodium nitrate at this time. It

is suggested that nitrites play a significant part in the so-called "grass tetany" of cattle, which is usually associated with herbage of high inorganic nitrogen content.

Grass treated with sodium nitrite at the rate of 3 cwt. per acre showed no visible signs of distress, although chemical analysis of the herbage gave evidence of metabolic disturbance.

The possible significance of nitrite in the synthesis of protein is briefly discussed.

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REFERENCES.

Baly, Heilbron and Hudson (1922). *J. Chem. Soc.* **121**, 1078.
Berthelot and Gaudechon (1911). *Compt. Rend. Acad. Sci.* **152**, 522.
Corbet (1934). *Biochem. J.* **28**, 1575.
Dhar, Bhattacharya and Biswas (1933). *Soil Sci.* **35**, 281.
—— and Mukherjee (1934). *Nature*, **134**, 499.
Eckerson (1924). *Bot. Gaz.* **77**, 377.
Eggleton (1934). *J. Agric. Sci.* **24**, 416.
Mevius and Dikussar (1930). *Jahrb. wiss. Bot.* **73**, 633.
Rimington and Quin (1933). *Onderstepoort J. Vet. Sci. and Animal Husbandry*, **1**, 469.
Schimper (1888). *Bot. Z.* **46**, 65.

CLXXI. THE DETERMINATION OF FRUCTOSE IN BLOOD.

By JOCELYN PATTERSON.

From the Department of Biochemistry, Charing Cross Hospital, Institute of Pathology.

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In direct determinations of blood fructose by colorimetric means, use has generally been made either of the original Selivanoff reaction [Folin and Berglund, 1922; Roe, 1934], or of the van Creveld [1927] reaction [Kronenberger and Radt, 1927; Radt, 1928; Corley, 1929] which employs diphenylamine. The present communication is concerned with the latter reaction only, which was investigated with the main object of comparing the results obtained with those resulting from the application of a new principle worked out in this laboratory [Scott, 1935].

Preliminary trials of the method described by Radt [1928] as suitable for the estimation of blood fructose furnished evidence which seemed to make it desirable to modify it in certain directions. The first unsatisfactory feature is that much more amyl alcohol is required to extract the coloured product in the treated blood filtrate than is required to carry out the corresponding operation upon the standard solution, and this has the effect of upsetting the volume relationships which it is desirable to maintain between unknown and standard. It was found that the extraction was greatly facilitated by the introduction of a little solid ammonium sulphate, which process enabled the final colour in both standard and unknown to be contained in the same volume.

The second important drawback to the method lies in the choice of blood filtrate. When using whole blood the filtrate from protein, precipitated with HCl and mercuric chloride, contains substances which interfere with the colour reaction between fructose and diphenylamine and have the effect of causing the development of colour in the unknown to proceed faster than in the standard and eventually to reach an intensity beyond that which could be attributed to the fructose present. This difficulty was readily overcome by a suitable choice of filtrate, for recent work on blood sugar has provided a number of filtrates established as satisfactory for true sugar determination, and though these are much more dilute than is advisable for the estimation of the very minute amounts of fructose that appear in blood after the ingestion of such quantities of the sugar as are commonly used in tolerance tests, the subsequent concentration was an operation easily accomplished without danger of vitiating the estimation.

On general grounds a zinc filtrate appeared to be eminently suitable for the purpose, and in that the form of precipitation used by Scott gave the opportunity of making this work strictly comparable with his, and further provided a greater volume of filtrate than that available from the standard Somogyi method, exactly the same procedure was adopted as that forming the preliminary stage to the bile salt colorimetric method. The quantities used were identical with those described as being applicable to a determination upon 2 ml. whole blood.

The subsequent stages of the estimation were carried out as follows. After the acidification with acetic acid the 10 ml. filtrate (equivalent to 1 ml. blood) were evaporated down (using a small Bunsen flame for direct heating) in a test-tube graduated at 4 ml. until the volume fell to just below the mark. It was then made up exactly to 4 ml. volume. This concentrated filtrate was then treated with 4 ml. HCl (5 parts concentrated acid + 3 parts distilled water), and 0·4 ml. 20 % alcoholic diphenylamine, the mixture shaken thoroughly, and then heated in a boiling water-bath for 15 minutes. On cooling to room temperature, 10 ml. butyl alcohol were added, followed by 2 g. solid ammonium sulphate. The tube was then stoppered and shaken up vigorously to permit extraction of the blue colour by the organic solvent. A solution of fructose (that containing 0·1 mg. fructose in the 4 ml. volume was the most suitable standard for use in tolerance tests) was similarly treated, and the butyl alcoholic layers were pipetted off for comparison in the colorimeter. Blank determinations on normal fasting bloods showed only the slightest trace of colour, which might for practical purposes be regarded as negligible. Actually it would appear to be equivalent to about 1 mg. fructose per 100 ml. blood, from the values recorded in Table I, which shows a comparison between the results ascertained by the process just outlined, and those obtained by the bile salt method.

Table I.

Sample	Estimated fructose mg./100 ml. Present method	Estimated fructose mg./100 ml. Scott's method	Type of case
1. With added fructose	11	10	Normal
2. With added fructose	16	15	Normal
3. 1 hour after fructose	13	12	Cirrhosis of liver
4. 2 hours after fructose	9	8	Cirrhosis of liver
5. 1 hour after fructose	19	18	N.A.B. Jaundice

The satisfactory agreement between the two sets of values establishes confidence in both methods as a means of determining blood fructose.

REFERENCES.

Corley (1929). *J. Biol. Chem.* **81**, 81.
van Creveld (1927). *Klin. Woch.* **6**, 697.
Folin and Berglund (1922). *J. Biol. Chem.* **51** 213.
Kronenberger and Radt (1927). *Biochem. Z.* **190**, 161.
Radt (1928). *Biochem. Z.* **198**, 195.
Roe (1934). *J. Biol. Chem.* **107**, 15.
Scott (1935). *Biochem. J.* **29**, 1012.

CLXXII. QUANTITATIVE STUDIES ON THE RESPONSE OF THE CAPON'S COMB TO ANDROSTERONE.

By ALAN WILLIAM GREENWOOD, JANET SCOTT SALMON BLYTH AND ROBERT KENNETH CALLOW.

From the Institute of Animal Genetics, University of Edinburgh, and the National Institute for Medical Research, London, N.W. 3.

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I. *Introduction.*

SINCE it was first reported by Pézard in 1911 that injection of extracts of testicular substance restored the characteristic sexual phase of growth in the comb of the castrated bird, this response has been used extensively to determine the efficacy of preparations containing the male hormone. A review of the literature to 1931 on the assay of male hormone will be found in the article by Koch [1932].

The earlier work of Gallagher and Koch and their co-workers, 1928–1930, [*cf.* Koch, 1932] showed that the marked individual variations in the responses of the capons to the hormone were not determined by such factors as age, weight of birds and initial size and shape of comb. This lack of influence of body weight and initial comb size on the extent of the reaction was confirmed by Blyth *et al.* [1931] but not by Freud *et al.* [1932]. It was found however that the effect of these individual variations could be minimised by previously selecting the birds and by using a number for each test.

Gallagher and his co-workers defined a bird unit as the amount of hormone which, when injected daily for 5 days, yields an average of 5 mm. increase in length *plus* height of the combs of at least 5 Brown Leghorn capons. In the studies recorded here the American procedure has been followed mainly for the reason that considerable familiarity with this method over a long period of time has led, in our opinion, to results which are as free from obvious irregularities as can be expected of any biological measurement.

Another method of estimating the amount of comb growth induced is that in use in the Laqueur laboratory where the increase in total area of the comb is determined by means of planimetric measurements of the photographic shadow. On this basis the unit of male hormone has been defined as one fourth of the smallest amount which, administered in 8 doses of 0·5 ml. in 4 successive days (morning and evening) subcutaneously or intramuscularly in oil solution, produces on the fifth day, in at least 2 of a group of 3 animals, a comb increase of at least 15 %, or if more than 3 birds are used, in the majority of the animals. Reference may also be made to the methods described by Schoeller and Gehrke [1931], Butenandt and Tscherning [1934], and Ruzicka and Tschopp [Ruzicka *et al.*, 1934].

The standardisation of preparations of male hormone by the comb growth method, like other biological assays, is unsatisfactory in that experiments carried out in different laboratories with the same technical procedure and the same hormone preparation do not necessarily give the same response. (Results of an international experiment carried out in 1933 under the direction of Koch in the laboratories of Laqueur, Koch, Butenandt and Greenwood (unpublished).) The discovery of a method for the artificial preparation of the male hormone, androsterone, by Ruzicka *et al.* [1934; 1935] and its production in larger quantity has suggested an extensive examination of the variables involved in the reaction between the hormone and the comb.

II. *Material and methods.*

Androsterone. This material was prepared in London by the method described by Ruzicka *et al.* [1934] with certain minor modifications [*cf.* Callow and Deanesly, 1935]. Material recrystallised from ethyl acetate and having a melting-point not below 182° was used. Solutions in arachis oil for injection were prepared by warming on the water-bath.

The stability of the oil solution was tested by heating a sealed ampoule containing the solution in an oil-bath, the temperature of which was thermostatically controlled at 149–151°, for 24 hours.

Care of capons. The birds on which these observations have been made were bred and kept at the Institute of Animal Genetics. They are housed under uniform conditions in a roof-lit, brick poultry house divided into pens each capable of holding 16–20 birds; the fowls are given access daily to floored sun porches but never come in contact with the ground.

Husbandry has been standardised as far as possible; the composition of the food remains the same throughout the year and consists of grain and mash in the summer months, while in the winter-time a hot mash is added. Cod-liver oil, green stuff, minerals and grit are also provided to supply the necessary special food requirements which cannot be picked up as on free range.

From October to March lighting is switched on in the evening until 6 o'clock and in the summer the birds are confined to the building from this time until 9 a.m. No artificial heating is provided.

Routine observations of the size of comb and body weight are made regularly. For the present series of experiments altogether 63 Brown Leghorn capons have been used, varying in age from 4 months to 6 years. The age at which the birds were castrated varied considerably as shown in Table I.

In preparation for the experiments measurements of the birds' combs and body weights were made (18. ii. 35) and 40 of the large group of 2-year-old castrates divided up into lots of 5 so that the average comb size and body weight of each was similar (see Tables II and III).

Table I. *Age at castration in different age groups.*

Prefix to no. of bird	Hatched	No. of birds	Average age at castration days	Range in days
A	1929	4	25	8–54
B	1930	4	26	18–33
D	1932	5	47	29–51
E	1933	45	39	21–51
G	1935	5	42	Nil

Comb measurement. All the birds were measured at the time of first injection and the differences in the average comb size (length *plus* height) of the groups from the preliminary measurement varied from $-0{\cdot}2$ to $+0{\cdot}8$ mm.

Since it appears that the technique of comb measurement employed by various operators does not yield strictly comparable results, a fairly detailed description of the procedure used in Edinburgh will be given. The measurements of greatest length and greatest height of the comb were made by means of a small pliable celluloid scale graduated in mm., and all data recorded were obtained from the measurements made by a single observer. The determination of the height of the comb usually presents no great obstacles, though very occasionally it is difficult to obtain a definite base line between the comb and the head. The length however is a different matter: as an anterior point from which to measure, the junction of the comb with the beak is not satisfactory since it fairly frequently happens that the bird damages this region with the result that the fleshy tissue retracts, and, if this occurs during an experiment a source of error is introduced into the results. While in a few of the capons the front of the comb runs down in an unbroken line to meet the beak, in most there is a slight transverse groove towards the front, and this wherever possible has been used as the anterior limit. In the normal cock the comb ends in a large blade extending posteriorly, but in the capon this part of the undeveloped comb frequently curves upwards so that its upper edge lies parallel with the last point of the comb; when growth is initiated it begins to move through an arc towards the position it would occupy in the normal cock, so that the line of greatest length from a fixed anterior point would not fall consistently on the same posterior point. In order to minimise the error in these cases, the initial measurement is made with the posterior blade of the comb pulled down to the position it would subsequently occupy. Apart from this manipulation the comb is not abnormally stretched in measuring; an attempt is merely made to get it lying as flat as possible between the points to be measured.

The injections were made at approximately the same time each day, and all groups of birds, with one exception, received the hormone preparation deep into the pectoral muscles, alternate injections being given in the left and right sides. The exceptional group received subcutaneous injections.

In all cases where small doses were given daily (0·2 ml.) the substance was administered by means of a 1 ml. tuberculin syringe graduated in 0·01 ml.

During the course of the experiments the combs were measured normally 3 and 5 days after the initial injection was given but, exceptionally, daily measurements were made to which special reference will be made later. The rate of comb regression following the cessation of injections has been determined for some groups by measurements up to 30 days from the initial injection.

III. *Examination of variables.*

Relation between dose and response. The first experiment, on the relation between the amount of hormone injected and the magnitude of the comb response, was performed on a series of 25 capons divided into groups of 5, each bird receiving daily injections of 0·2 ml. of arachis oil solution for 5 days. The amount of hormone given was doubled in each successive group, and the total dose per bird ranged from 0·5 to 8 mg. of androsterone.

The data are recorded in Table II and plotted in Fig. 1. The individual points on the graph show that the variability of the response to the different doses is very similar. Thus, for the mean responses in the five groups receiving 0·5, 1, 2, 4 and 8 mg. total dose, σ is respectively 3·0, 1·9, 2·3, 2·5 and 1·8. This means that the relative variability (coefficient of variation, or σ/mean × 100) is much less for the larger responses than for the smaller ones, which suggests that for comparative work it is desirable to aim at a substantial response. In

Table II.

Series A. Dose/response data. (Figs. 1, 2 and 6.)

No. of bird	Body weight g.		Comb size (mm.)				Increase in 3 days	Increase in 5 days	Comb size (mm.)					
	18. ii. 35	4. iii	18. ii	27. ii	2. iii	4. iii			6. iii	9. iii	14. iii	19. iii	24. iii	29. iii
Group 1.	Total dose 0·5 mg.													
E. 99	2350	2400	66	66	68	74	2	8	73	72	69	69	69	69
214	2850	2800	71	70	69	71	-1	1	72	70	69	68	68	67
232	3300	3250	67	71	71	72	0	1	72	71	71	70	71	68
399	2800	2850	60	61	63	64	2	3	64	62	62	61	61	60
533	2550	2600	75	75	75	76	0	1	76	75	76	74	74	73
Average	2770	2780	67·8	68·6	69·2	71·4	0·6	**2·8**	71·4	70·0	69·4	68·4	68·6	67·4
Group 2.	Total dose 1 mg.													
E. 153	3200	3200	73	73	75	78	2	5	77	76	76	75	74	73
182	2700	2700	61	61	63	67	2	6	67	65	64	65	64	62
275	2900	2900	70	71	73	80	2	9	79	76	76	74	74	75
330	3150	3150	80	80	82	87	2	7	88	85	84	85	81	83
653	1950	1950	55	56	57	60	1	4	61	59	58	56	56	56
Average	2780	2780	67·8	68·2	70·0	74·4	1·8	**6·2**	74·4	72·2	71·6	71·0	69·8	69·8
Group 3.	Total dose 2 mg.													
E. 10	2200	2200	77	78	86	91	8	13	90	87	86	85	85	85
227	2650	2650	53	54	58	61	4	7	61	59	59	58	58	57
317	3400	3500	74	72	78	84	6	12	84	82	81	78	77	78
350	2650	2700	65	64	69	74	5	10	74	72	71	71	70	70
403	3050	3100	70	70	74	81	4	11	79	76	74	71	71	70
Average	2790	2830	67·8	67·6	73·0	78·2	5·4	**10·6**	77·6	75·2	74·2	72·6	72·2	72·0
Group 4.	Total dose 4 mg.													
E. 126	2050	2100	51	53	63	70	10	17	69	65	63	63	63	63
145	3200	3250	76	76	82	90	6	14	91	88	87	84	83	83
187	3400	3400	74	75	80	89	5	14	88	87	84	81	80	81
328	2600	2600	66	63	74	82	11	19	82	76	74	76	75	74
385	2700	2700	71	70	77	83	7	13	84	81	80	76	75	74
Average	2790	2810	67·6	67·4	75·2	82·8	7·8	**15·4**	82·8	79·4	77·6	76·0	75·2	75·0
Group 5.	Total dose 8 mg.													
E. 24	2650	2600	75	76	87	93	11	17	93	89	88	87	86	86
230	2700	2800	68	69	80	86	11	17	87	85	82	79	78	79
412	2900	2950	71	69	79	89	10	20	89	84	83	81	79	78
536	2900	2850	63	64	74	82	10	18	81	78	76	74	74	74
585	2900	2850	62	64	71	79	7	15	80	76	74	73	72	72
Average	2810	2810	67·8	68·4	78·2	85·8	9·8	**17·4**	86·0	82·4	80·6	78·8	77·8	77·8

(Daily dose: 0·2 ml.)

view of the constant magnitude of the variability of the response with different doses it is legitimate to consider the whole of the 25 observations together and to calculate a combined σ. The figure obtained is 2·1, which may be taken as the standard deviation of the comb response under the conditions and range of dosage used. The standard error of the response may therefore be taken as ± 1·0

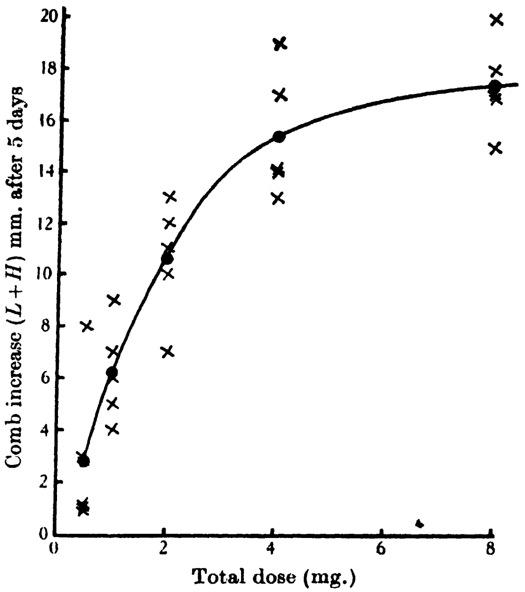

Fig. 1. Relation of increase of size of comb to the total dose of androsterone given over 5 days. Series A. × Individual birds; • mean values for groups of 5.

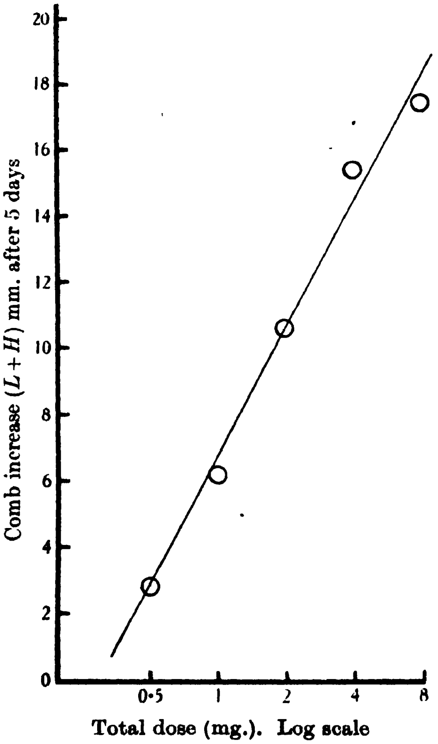

Fig. 2. Relation of increase of size of comb to log (total dose).

for a group of 5 capons, or ±0·7 for a group of 10 capons. In comparing the responses in two groups of 5 therefore a difference of not less than 3·0 mm. is required in order to be statistically significant.

The curve drawn through the means in Fig. 1 obviously gives the elements of a standardisation curve; its shape suggests that the relation between dose and response is logarithmic, and when, as in Fig. 2, the response is plotted against the logarithm of the dose, it is seen that the points lie approximately along a straight line. The observations embodied in this (log dose)/(response) line were all made during the same week, and it is well known that such lines vary from time to time in both position and slope—an important factor in assay work. They can, however, always be reorientated by giving two different doses of a standard preparation at the same time as an assay is made.

From the line in Fig. 2 we obtain graphically $\frac{du}{dx}=\frac{12\cdot 8}{1}$, where u = comb response in mm. and x = logarithm of the dose in mg. The standard error of ±1·0 in the mean response of a group of 5 capons will thus represent an error of about ±0·08 in the determination of the logarithm of the dose. This is equivalent to an error of about ±18 % in the determination of the dose. With groups of 10 capons the error would be reduced to about ±12 %.

Nature and volume of medium. Two tests were designed to examine the possible influence of changes in the medium in which the hormone is dissolved. In series B (Table III and Fig. 3) the actual amount of hormone injected daily was the same as in group 3 of the first experiment, namely 0·4 mg., but the volume of oil in which it was administered was increased from 0·2 to 1·0 ml. In series D (Table III and Fig. 3) the amount of hormone was again kept the same but the medium altered, both in nature and quantity, being a solution in 10 % aqueous alcohol, given in a daily dose of 4 ml. Five birds were used for each test, their average comb size and body weight being approximately the same as those for the groups in series A. An examination of the responses obtained in these two experiments, compared with that obtained from a similar amount of hormone injected into group 3 of the first experiment shows that the increase in comb size of 12·4 mm. resulting from the administration of the aqueous-alcoholic solution (series D) is nearly twice that obtained in series B (7·4 mm.) with the increased volume of oil, while that in series A (which will be shown later to approximate closely to the average of all increases obtained from this dosage) is intermediate between them.

It is clear then that in any procedure for the standardisation of the hormone it is necessary to consider not only the nature of the solvent employed but also the volume.

Site of injection. In the next experiment (series C, Table III and Fig. 3) the same dosage was adopted as in A 3, but the injections were given subcutaneously instead of intramuscularly. It will be seen from Fig. 3 that the reactions obtained in the two experiments follow similar courses, the response elicited by subcutaneous injection being slightly but not significantly less than by intramuscular injection.

Heat stability of androsterone. Data concerning the effect of subjecting the hormone to heating at 150° for 24 hours are to be found in Table IV. Control and heated solutions of androsterone were administered in the same concentration and dosage as in A 3. The birds in this experiment had been used previously (series C and D respectively) and, 17 days after the final injection, the comb size in both groups had regressed to approximately the same extent.

Table III. (*Fig. 3.*)

No. of bird	Body weight g.	Comb size (mm.)				Increase in		Comb size (mm.)			
		18.ii.35	9.iii	12.iii	14.iii	3 days	5 days	16.iii	19.iii	24.iii	29.iii
Series B. Effect of volume of oil medium.											
0·4 mg./ml. Total dose 2 mg.											
E. 165	2650	57	58	60	61	2	3	62	62	62	60
304	2900	67	67	73	76	6	9	78	75	74	71
325	3400	73	74	80	84	6	10	83	82	82	81
327	2650	70	71	76	80	5	9	81	76	77	75
346	2450	72	70	73	76	3	6	75	74	72	69
Average	2810	67·8	68·0	72·4	75·4	4·4	**7·4**	75·8	73·8	73·4	71·2
Series C. Effect of subcutaneous injection.											
2·0 mg./ml. Total dose 2 mg.											
E. 144	3050	69	69	73	78	4	9	77	74	74	73
156	3200	73	72	75	81	3	9	82	81	80	79
274	2500	62	63	68	73	5	10	73	68	66	65
640	3050	64	66	71	78	5	12	78	76	74	73
902	2300	71	72	76	80	4	8	78	77	76	75
Average	2820	67·8	68·4	72·6	78·0	4·2	**9·6**	77·6	75·2	74·0	73·0
Series D. Effect of aqueous-alcoholic medium.											
0·1 mg./ml. Total dose 2 mg.											
E. 11	1900	68	70	74	81	4	11	83	82	77	75
75	3100	68	69	74	82	5	13	85	86	83	80
77	3200	72	72	78	87	6	15	85	83	82	82
102	2600	66	64	69	74	5	10	73	71	70	68
244	3400	65	66	74	79	8	13	77	76	75	74
Average	2840	67·8	68·2	73·8	80·6	5·6	**12·4**	80·6	79·8	77·4	75·8

Note. Series B, C and D may be compared with group 3, Series A (Table II), where the amount of androsterone injected was the same.

Table IV.

Series E. Effect of heating androsterone in oil solution.

No. of bird	Comb size (mm.)				Increase in		
	30.iii.35	1.iv	2.iv	4.iv	2 days	3 days	5 days
Group 1. Heated solution. Total dose 2 mg.							
E. 165	60	66	68	72	6	8	12
304	71	75	79	83	4	8	12
325	81	84	87	93	3	6	12
327	75	81	83	88	6	8	13
346	69	72	75	77	3	6	8
Average	71·2	75·6	78·4	82·6	4·4	7·2	**11·4**
Group 2. Control. Total dose 2 mg.							
E. 144	73	77	78	83	4	5	10
156	79	82	87	93	3	8	14
274	65	70	74	81	5	9	16
640	73	77	80	84	4	7	11
902	75	79	81	83	4	6	8
Average	73·0	77·0	80·0	84·8	4·0	7·0	**11·8**

In this case the close parallelism between the comb responses with control and heated solutions shows definitely that the latter solution had not lost activity.

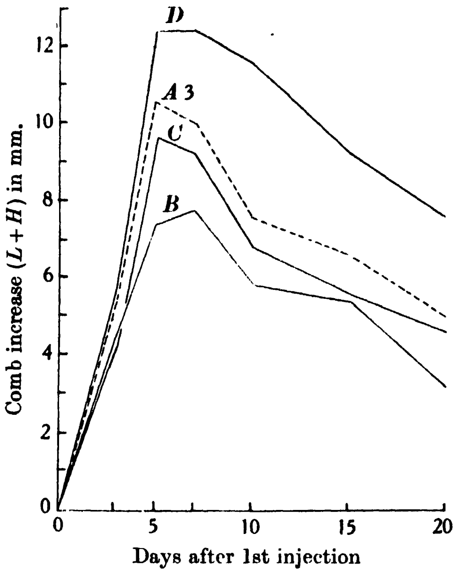

Fig. 3.

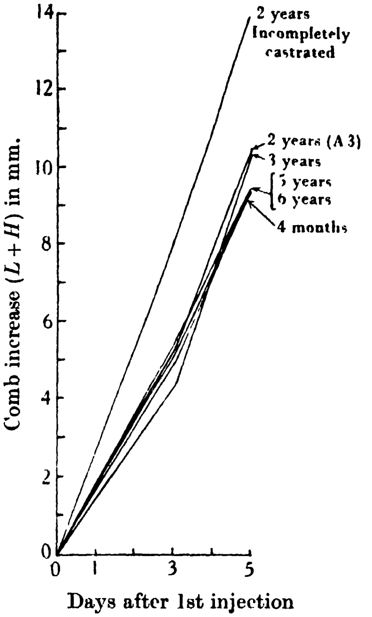

Fig. 4.

Fig. 3. Growth and regression of combs under varying conditions of administration of androsterone (2 mg. in 5 days). Groups of 5 birds. A 3, control; B, dilute oil solution; C, subcutaneous injection; D, aqueous alcoholic solution.

Fig. 4. Effect of age of bird and of incomplete castration (upper curve) on comb growth. (Dose 2 mg. in 5 days.) Series G.

Age of bird. The possible effect of age of the capon on the amount of comb-response induced was considered in the next experiment. Age groups of 2, 3, 5 and 6-year-old capons as well as one of 4 months were subjected to the same treatment as group A 3 (0·2 ml. daily for 5 days of a solution containing 2 mg. of androsterone per ml.). The small number of older birds available from which to select subjects for these tests made it necessary to include only 4 birds in each of the two oldest groups instead of five as in other experiments. The 2-year-old birds, group G 4, included in this test had been previously discarded, because, although the combs in point of view of size fell well within the range of the completely castrated birds their appearance and colour suggested that castration had not been satisfactorily complete. This suspicion was confirmed when the results of the test became available and it was seen that, whilst the responses obtained from the other age groups fell relatively closely to one another, that from the 2-year-old group was significantly larger (Table V and Fig. 4).

If, however, series A 3, which is more strictly representative of the 2-year-old birds, is used for comparison, it is found that the increase obtained after 5 days is just above that of the 3-year-old group (10·6 mm. to 10·4 mm. respectively) which in turn exceeds somewhat the increase elicited in the remaining three groups; these small differences are not significant.

Table V. (*Fig. 4.*)

Series G. Effect of age of birds.

No. of bird	Comb size (mm.)			Increase in		Body weight g.
	29. iii. 35	1. iv	3. iv	3 days	5 days	29. iii
Group 1. 6 years old						
A. 292	70	75	79	5	9	2750
333	76	80	84	4	8	3050
462	79	84	87	5	8	2650
695	63	71	76	8	13	3000
Average	72·0	77·5	81·5	5·5	**9·5**	2862
Group 2. 5 years old						
B. 599	63	65	69	2	6	2100
641	65	71	77	6	12	3050
687	59	69	75	10	16	2650
974	81	83	85	2	4	3000
Average	67·0	72·0	76·5	5·0	**9·5**	2700
Group 3. 3 years old						
D. 129	72	77	85	5	13	2950
130	63	67	71	4	8	3400
456	69	74	81	5	12	3100
935	71	74	77	3	6	2850
953	66	71	79	5	13	2900
Average	68·2	72·6	78·6	4·4	**10·4**	3040
Group 4. 2 years old						
E. 5	71	81	88	10	17	2300
270	78	86	92	8	14	2700
552	71	76	82	5	11	2600
679	77	86	92	9	15	3150
712	71	79	84	8	13	2400
Average	73·6	81·6	87·6	8·0	**14·0**	2630
Group 5. 4 months old						
	11. iv. 35	14. iv	16. iv			11. iv
G. 1	64	68	73	4	9	1400
—	72	77	81	5	9	1850
3	71	77	81	6	10	1450
4	64	70	73	6	9	1400
5	69	74	79	5	10	1700
Average	68·0	73·2	77·4	5·2	**9·4**	1560
Av. for all groups	69·8	75·4	80·4	5·6	**10·6**	2540

(Total dose: 2 mg. of androsterone, 2 mg./ml.)

Weight of birds. If the possible effect of initial body weight on the magnitude of the comb response is examined it might be considered on *a priori* grounds that the concentration of the hormone in the body fluids is a factor in determining the amount of response and that therefore an inverse relationship would exist between the comb increase obtained and the body weight of the bird. However, no such relationship can be discovered in our experiments. Moreover, in the age series (Table V) the 4-months-old group, which has an average body weight of 1560 g. compared with an average body weight of from 2630 to 3040 g. in the older groups (2 years to 6 years old), produced a comb increase which was not above the average for the dosage and indeed was slightly lower.

Initial size of comb. An analysis of the responses obtained from the relatively large number of 2-year-old capons with a view to determining whether any relation exists between the initial comb size and the magnitude of the comb reaction, in previously untreated birds, leads, if we exclude the age group G 4 for reasons explained above, to the conclusion that no consistent relation exists between them.

Previous treatment. In order to determine the effect of previous treatment on the magnitude of subsequent responses, 26 days after the cessation of the first experiment all the birds in series A were subjected to a further series of androsterone injections. The dosage was the same in all groups, being a daily injection of 0·2 ml. of a solution containing 2 mg. of the hormone per ml. On the previous occasion only one group (A 3) received this dosage, two of the others (A 1 and A 2) having had lower doses and two higher (A 4 and A 5).

When these results (Table VI and Fig. 5) are examined for evidence of any "priming" effect, it is found that 25 capons receiving 2 mg. gave an average increase in comb size of 11·3 mm., compared with 10·6 mm. given by a group of 5 birds a month before; the difference is not significant. The maximum differences between the groups are barely significant, and any correlation with the degree of previous priming is disposed of by the low response in group 5, unless it can be speculatively suggested that the responses in groups 3 and 4, which show a difference on the verge of statistical significance, indicate an optimum priming effect.

Rate of comb growth. The rate of growth of the combs in the first experiment (Table II and Fig. 6) where different amounts of androsterone were used, presented no exceptional features. When comb measurements were made 3 days after the initial injection it was found that the increase in comb size was roughly related to the amount of hormone injected. The measurements at 5 days indicated that increased responses were obtained with increasing dosage in the growth period between 3 and 5 days except in the case of the maximum dosage where the response to the two highest doses was similar. The lack of a directly proportional response of the comb to the amount of hormone injected is suggested by the amount of growth in the various groups at 3 days but is well marked in the amount of growth occurring in the succeeding period.

In this series, where the comb measurements were recorded after 3 and 5 days from the initial injection, a point of note is that those groups receiving the lower doses showed a distinct lag in response during the first period, suggesting that a definite latent period exists before the influence of the hormone can be expressed in the comb, or that a threshold of stimulus had to be attained before the hormone could prove effective. (The stimulation provided by the higher doses was apparently sufficient rapidly to overcome this inhibition or to mask it.) In this respect the data for the daily measurements at the second test of this series (Table VI) are of interest. As was expected with the moderate dosage used the increase obtained 24 hours after the initial injection was small and between 24 and 48 hours comparatively large. On the 4th and 5th days the daily increment in the size of the comb approached that obtained at 48 hours, but on the 3rd day the figure recorded was distinctly low. Since there appears no obvious reason why such a lag in the amount of the response should occur at this time the question arises whether it can be explained by errors in measure-

Table VI.

Series F. Effect of previous treatment. (*Fig. 5.*)

No. of bird	Comb size (mm.)								Increase in days						
	29.iii.35	30.iii	31.iii	1.iv	2.iv	3.iv	4.iv	5.iv	1	2	3	4	5	6	7
Group 1															
E. 99	69	70	74	77	81	84	84	81	1	5	8	12	15	15	12
214	67	68	70	72	77	77	79	78	1	3	5	10	10	12	11
232	68	69	72	72	74	75	77	76	1	4	4	6	7	9	8
399	60	61	64	64	67	69	69	69	1	4	4	7	9	9	9
533	73	73	76	78	78	80	81	79	0	3	5	5	7	8	6
Average	67·4	68·2	71·2	72·6	75·4	77·0	78·0	76·6	0·8	3·8	5·2	8·0	**9·6**	10·6	9·2
							Daily increase			3·0	1·4	2·8	1·6	1·0	−1·4
Group 2															
E. 153	73	72	75	76	77	80	80	78	−1	2	3	4	7	7	5
182	62	64	66	66	69	72	72	71	2	4	4	7	10	10	9
275	75	75	79	81	83	89	88	87	0	4	6	8	14	13	12
330	83	83	86	88	91	94	95	95	0	3	5	8	11	12	12
653	56	56	60	60	62	64	64	63	0	4	4	6	8	8	7
Average	69·8	70·0	73·2	74·2	76·4	79·8	79·8	78·8	0·2	3·4	4·4	6·6	**10·0**	10·0	9·0
							Daily increase			3·2	1·0	2·2	3·4	0·0	−1·0
Group 3															
E. 10	85	88	92	96	99	101	101	98	3	7	11	14	16	16	13
227	57	58	61	62	64	66	68	66	1	4	5	7	9	11	9
317	78	79	84	87	90	95	96	93	1	6	9	12	17	18	15
350	70	70	73	74	78	80	79	77	0	3	4	8	10	9	7
403	70	71	73	76	79	82	82	81	1	3	6	9	12	12	11
Average	72·0	73·2	76·6	79·0	82·0	84·8	85·2	83·0	1·2	4·6	7·0	10·0	**12·8**	13·2	11·0
							Daily increase			3·4	2·4	3·0	2·8	0·4	−2·2
Group 4															
E. 126	63	64	70	73	76	80	80	79	1	7	10	13	17	17	16
145	83	83	87	88	92	93	96	94	0	4	5	9	10	13	11
187	81	81	83	84	88	91	92	89	0	2	3	7	10	11	8
328	74	75	79	81	83	86	88	84	1	5	7	9	12	14	10
385	74	75	78	80	83	88	89	85	1	4	6	9	14	15	11
Average	75·0	75·6	79·4	81·2	84·4	87·6	89·0	86·2	0·6	4·4	6·2	9·4	**12·6**	14·0	11·2
							Daily increase			3·8	1·8	3·2	3·2	1·4	−2·8
Group 5															
E. 24	86	88	93	95	98	100	101	98	2	7	9	12	14	15	12
230	79	81	84	84	87	92	91	88	2	5	5	8	13	12	9
412	78	80	82	84	86	87	88	89	2	4	6	8	9	10	11
536	74	75	77	80	84	85	85	83	1	3	6	10	11	11	9
585	72	72	74	76	79	82	82	80	0	2	4	7	10	10	8
Average	77·8	79·2	82·0	83·8	86·8	89·2	89·4	87·6	1·4	4·2	6·0	9·0	**11·4**	11·6	9·8
							Daily increase			2·8	1·8	3·0	2·4	0·2	−1·8
Total av. (25 birds)	72·4	73·2	76·5	78·2	81·0	83·7	84·3	82·4	0·8	4·1	5·8	8·6	11·3	11·9	10·0
							Total daily increase			3·3	1·7	2·8	2·7	0·6	−1·9

(Total dose: 2 mg. of androsterone, 2 mg./ml.)

ment, but this seems unlikely since for this reading the amount of increase recorded is consistently low for all the groups. (It is true that in group 3 it approaches a higher level but equally true that it is lower than the daily observations made immediately preceding and following it.) Whatever its meaning, the observations made force us to the conclusion that it is a real phenomenon.

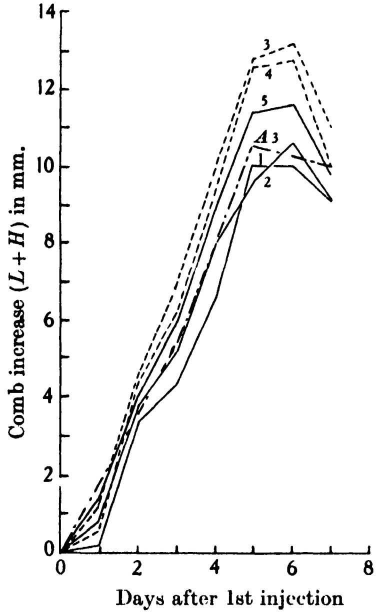

Fig. 5.

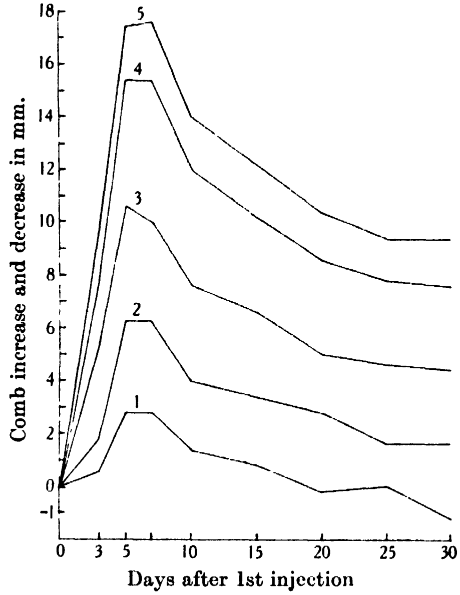

Fig. 6.

Fig. 5. Effect of previous treatment on growth of comb of groups of 5 birds receiving 2 mg. of androsterone over 5 days. Series F.

Fig. 6. Growth and regression of combs in groups of 5 birds receiving different doses. Series A. Group 1, 0·5 mg.; group 2, 1 mg.; group 3, 2 mg.; group 4, 4 mg.; group 5, 8 mg.

Rate of comb atrophy. The behaviour of the capon's comb after treatment has ceased may also be studied briefly in the same two tests. In series A measurements were made on the 7th and 10th days from the commencement of the experiment and thereafter at 5-day intervals for a further 20 days (Table II and Fig. 6). In series F the daily measurements were continued until the 7th day. For the former group the section of the curve under consideration may be conveniently divided into two parts, that between the 5th and 7th days, which shows comparatively little change, and the remainder of the curve, characterised by an initial phase of rapid regression which however is not maintained. The individual curves, though exhibiting some variability, suggest that the rate of regression decreases as the comb size becomes progressively smaller. The decline also appears to be greater in groups receiving the higher doses and this is particularly evident in the first sharp fall in the size of the comb (between the 7th and the 10th days). Nevertheless the inter-group divergence in regression rate is slight and it is clear that, whilst the combs of the first group have returned to their original state by the 20th day, a considerable time must elapse before those of the birds receiving the higher doses do so. In fact by the 30th day the average comb size in the birds of group A 5 had only regressed to the level attained by A 3 at its maximum.

With regard to that part of the curves between the 5th and 7th days, whereas A 3 exhibited a slight decrease the other groups remained stable at their maximum level. It is interesting to compare these results with those for series F. Here, daily measurements were recorded, and while group 1 showed a definite increase in the size of the comb on the 6th day, the others were more or less stable at the maximum size. By the 7th day however all had regressed to a size below that obtaining on the 5th day, the fall in the majority of the groups being greater than that in A 3.

SUMMARY.

1. A quantitative study has been made of the response of the comb of the Brown Leghorn capon to crystalline androsterone, and the effect of certain variables has been investigated.

2. In order to minimise the effect of individual variation, groups of five birds of similar initial comb size and body weight were used. These have been kept under constant conditions, and the technique of comb measurement has been carefully standardised.

3. Daily injections were made for 5 days, and the response was measured in terms of the increase in maximum length *plus* height of the comb.

4. The relation between the amount of hormone injected and the response of the comb measured on the day after the 5th injection is not linear when doses greater than 2 mg. of androsterone over 5 days are administered, corresponding to an increase of about 10 mm. in length *plus* height, but the response is directly proportional to the logarithm of the dose between the limits 0·5 and 8 mg. over 5 days.

5. The results are considered in relation to the formulation of a satisfactory technique for the assay of the male hormone. Careful measurement on selected groups of 5 birds yields results which imply a standard error of about ± 18 % in the determination of the dose.

6. The amount and nature of the solvent influence the response.

7. The responses to subcutaneous and to intramuscular injection are similar.

8. The differences in response with groups of widely differing ages (4 months to 6 years) are negligible.

9. It has been shown that in certain cases, probably as the result of the presence of a fragment of testis, unusually large responses are obtained. It is suggested that, as a precaution, birds should be used not earlier than 12 months after castration.

10. No relation between body weight and comb response could be demonstrated, nor could any consistent relation between initial comb size and amount of growth be found.

11. It is important to fix the period of the assay, since the amount of comb growth on successive days is not regular.

12. Only when a relatively small amount of comb growth is induced can regression of the comb to its pre-injection level be expected within a month, but after this period the responses to a new series of doses, whilst slightly irregular, do not show any significant effect of "priming".

13. The activity of androsterone is unaffected by heating the solution in arachis oil at 150° for 24 hours.

We are indebted to Dr A. S. Parkes for much useful advice and criticism in the course of this work.

REFERENCES.

Blyth, Dodds and Gallimore (1931). *J. Physiol.* **73**, 136.
Butenandt and Tscherning (1934). *Z. physiol. Chem.* **229**, 167.
Callow and Deanesly (1935). *Biochem. J.* **29**, 1424.
Freud, de Fremery and Laqueur (1932). *Pflüger's Arch.* **229**, 763.
Koch (1932). Sex and internal secretions. (Ed. E. Allen. Baltimore.)
Ruzicka (1935). *Naturwiss.* **23**, 44.
—— Goldberg, Meyer, Brüngger and Eichenberger (1934). *Helv. Chim. Acta*, **17**, 1395.
Schoeller and Gehrke (1931). *Wien. Arch. inn. Med.* **21**, 329; *cf. Zahnärztl. Mitt.* **13**, March 29th.

CLXXIII. GROWTH AND MAINTENANCE OF THE FOWL'S COMB BY ADMINISTRATION OF ANDROSTERONE.

By ROBERT KENNETH CALLOW and ALAN STERLING PARKES.
From the National Institute for Medical Research, London, N.W. 3.

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I. *Introduction.*

GREENWOOD *et al.* [1935] have discussed certain quantitative aspects of the capon comb response to androsterone. In view of the need for economy in both time and material a comparatively small degree of response is most suitable for assay work. Thus the criteria commonly used, a 20 % increase in area or an increase of 5 mm. in length + height, take the capon comb only a fraction of the way towards the size of the normal cock's comb. Owing to lack of material and preoccupation with assay work, comparatively little effort has hitherto been made to produce and maintain full-sized combs in capons.

Early in the work on the preparation of male hormone from testis, McGee *et al.* [1928] obtained comb growth in a Leghorn capon to a size ($L \times H$) of 48 cm.^{2} in 5 weeks, about half the area (thus calculated) for a really good Brown Leghorn cock comb. Schoeller and Gehrke [1931] obtained an increase to 40·3 cm.^{2} in 6 weeks with urine concentrates.

Ruzicka *et al.* [1934] obtained a surface area of 20 cm.^{2} (from 6·5 cm.^{2}) in 20 days during early work with their "synthetic" androsterone and Ruzicka and Tschopp [1935] record an increase from 4 to 27 cm.^{2} in 16 days. The record comb of the literature, however, appears to have been produced by Freud *et al.* [1932]. As a result of nearly 4 months' injection of male hormone concentrate from bull testis, ultimately in a dose corresponding to 6 kg. of testis daily, they obtained a comb of 83 cm.^{2} (planimeter reading), which may be considered as being full size. This problem is of importance because there is some reason to suppose that androsterone may not represent the full endocrine activity of the mammalian testis, and an examination of the requirements for the complete replacement of testicular activity in birds should be instructive.

We have also taken the opportunity of recording some observations on the effect of androsterone on the atrophic vas deferens.

II. *Methods and material.*

Birds. Two Brown Leghorn capons (EC 5 and 8) were obtained from the stock maintained by Dr A. W. Greenwood, having been operated on as chicks. One Brown Leghorn (EC 1) and two Plymouth Rocks (EC 2 and 3) were successfully caponised as adults and used for maintenance experiments. One Plymouth Rock (EC 7) and the Sebright Bantam were operated on as adults, and kept for more than a year before use, by which time the combs were indistinguishable from those of birds caponised as chicks. The difficulties of caponising adult cocks are considerable, and several birds were lost in obtaining reasonably clean operations. Two normal hens, one Rhode Island Red and one Brown Leghorn were also used.

Accommodation. The groups of capons discussed in Section VII were kept in an outside run open only to the north-east. The other birds were kept loose in an inside room, well lighted by two large windows, but receiving no direct sunlight.

Comb measurements. In the long period experiments combs were measured twice a week or, during periods of rapid growth, every day. In the comparative experiments described in Sections V and VIII the method of testing described by Greenwood *et al.* [1935] was used.

In order to give an adequate impression of the changes in comb size it is necessary to obtain either the surface area or the volume of the comb or an index of one of these. Length + height ($L+H$), whilst a convenient basis for assay work, does not indicate the actual changes in the appearance of the comb. We have therefore taken $L \times H$ as an index of comb size. The measurements (length × breadth in the case of the rose comb of the Sebright Bantam) were made as described by Hill and Parkes [1934].

Source of androsterone. The androsterone used in these experiments was part of the stock referred to by Callow and Deanesly [1935]. Whilst lower concentrations were used at the beginning, the bulk of the work was carried out with solutions of 10 mg./ml. arachis oil. A solution of this strength can readily be made by heating the oil, and the material afterwards remains in solution at room temperature. Stronger solutions in arachis oil can be made but they tend to crystallise out on cooling. A concentration of 10 mg./ml. represents about 100 capon units/ml. and makes it possible to keep the amount of oil injected down to a comparatively small quantity.

III. *Induction of full comb growth.*

Sebright Bantam capon. A Sebright Bantam cock was castrated in December 1933, when about 6 months old. The comb, 5·7 × 2·6 cm. at operation, atrophied rapidly, and 2 months later was 4·0 × 1·5 cm.; 3 months later it was 3·1 × 0·8 cm., at which it remained constant for 8 months. During this time the henny plumage was superseded by typically cocky plumage and spurs developed. 13 months after castration, injections of androsterone were begun, starting at

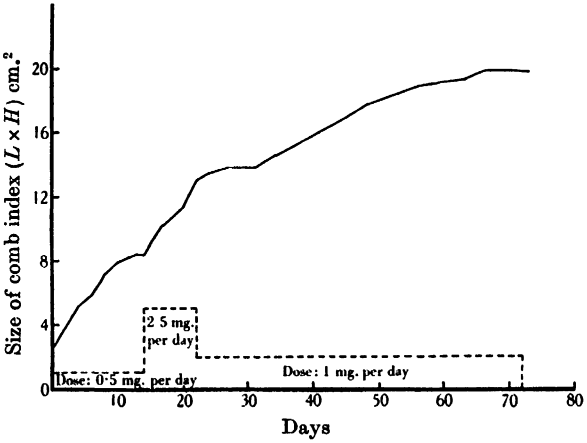


Fig. 1. Growth of comb in a Sebright Bantam capon.

0·5 mg., rising to 2·5 mg. and dropping again to 1·0 mg. daily. At the end of treatment, the comb was larger than at the time of castration and was in fact far larger than that of the normal cock. The treatment had no effect on the capon plumage [see Callow and Parkes, 1935]. The details of the experiment are shown in Fig. 1. It may be concluded that 0·5 mg. will cause appreciable growth of the comb of the Sebright capon, but will not take it to full size. 1·0 mg. daily, on the other hand, will grow the comb to abnormal size and will maintain it there. It may thus be supposed that the testes of the normal Sebright cock have a comb growth-promoting activity equivalent to that of rather more than 0·5 mg. androsterone administered as above.

Brown Leghorn capon. This bird was from Dr Greenwood's stock. The comb was flaccid and though rather large (5·7 × 2·7 cm.) when stretched, was brown and shrivelled and evidently that of a complete capon (Plate IV, fig. 7). It was thus ideal for the experiment in view, *viz.* the production of a full-sized comb in the minimum time. 5 mg. androsterone daily were given. Really remarkable growth was produced (Fig. 2), and after 22 days' treatment the comb measured 11·8 × 7·2 cm. and would readily have passed as that of a normal cock, except possibly that the papillae were not very marked (Plate IV, fig. 8). Wattles and

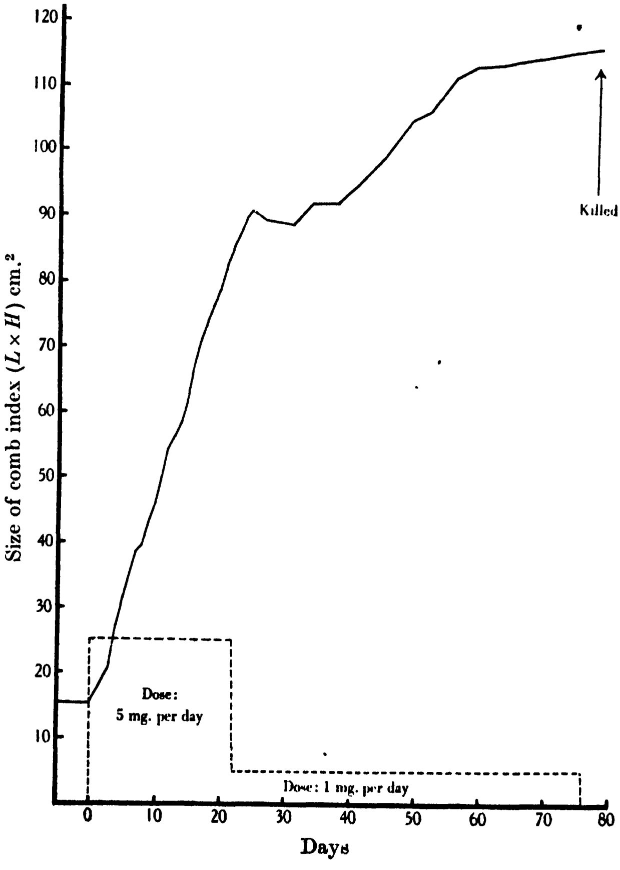


Fig. 2. Growth of comb in a Brown Leghorn capon.

ear lobes were also prominent. The dose was then dropped to 1 mg. daily, which was thought to be about the probable maintenance requirement. After a set-back, due probably to the sudden drop in dosage, growth was resumed, though at a much slower rate. This second growth phase gradually died away and in the end the comb was being comfortably maintained by administration of 1 mg. daily at a size which was above rather than below the usual standard for the breed. 10 days after the first injection this bird was observed to be making vigorous efforts to "tread" a hen, and a few days later it began to crow sporadically, a condition which was maintained till it was killed. This induction of "treading" and crowing is of interest as an effect of the administration of a pure crystalline substance. According to Appel [1929] the syrinx is morphologically similar in hens, capons and cocks, so that the power to crow presumably depends merely on the "instinct" to do so.

Comparison of Brown Leghorn and Plymouth Rock capons. The combs of Brown Leghorn cocks are usually 12–14 × 7–8 cm. Abnormally large ones run up to 16 × 9 cm., or even more. The cocks of heavy breeds of fowl in general have smaller combs. The combs of Plymouth Rock cocks maintained at the Institute's Field Laboratories, for instance, are usually only 9–10 × 4–5 cm., and they never attain such an appearance of luxuriant growth as those of Leghorns. It seemed of interest therefore to determine whether this difference is due to a difference in the amount of male hormone produced by the testes in the Plymouth Rocks, or to a greater sensitivity of the combs of the Leghorns. For this purpose, Leghorn and Plymouth Rock capons with combs similar

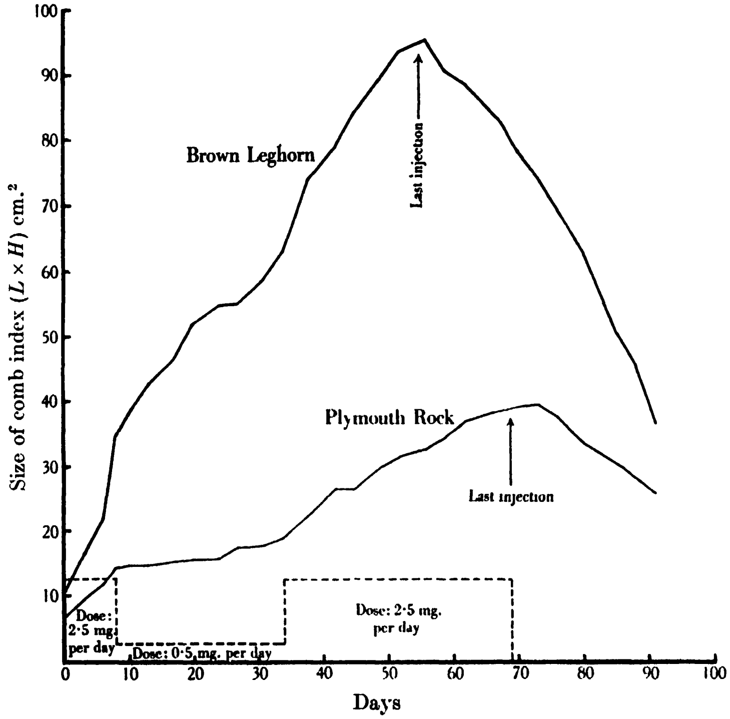


Fig. 3. Comparison of comb growth in a Brown Leghorn and a Plymouth Rock capon.

in size and shape were chosen. The Leghorn, again one of Dr Greenwood's stock, had a comb 4·7 × 2·2 cm. The Plymouth Rock, which had been castrated 14 months previously as an adult, had a comb which had been fairly constant at about 4·7 × 1·4 cm. for many months. These birds were each given 2·5 mg. daily for 8 days, then 0·5 mg. daily for 25 days and finally 2·5 mg. again (see Fig. 3). During the first 8 days the comb of the Leghorn more than tripled in $L \times H$; that of the Plymouth Rock just doubled. During the administration of 0·5 mg. daily, the Leghorn comb nearly doubled, that of the Plymouth Rock hardly grew at all. During the second period of administration of 2·5 mg. daily the Leghorn comb increased in $L \times H$ by 50 % in 21 days. The comb of the Plymouth Rock rather more than doubled in 35 days, but the actual increment was much less than in the case of the Leghorn. When injections were stopped the comb of the Plymouth Rock was rather larger than at the time of castration 16 months previously. As shown in Fig. 3 the differential rate of growth in these two birds was paralleled by the differential rate of atrophy after the last injection. This experiment suggests strongly that the difference in comb size in Leghorns and Plymouth Rocks is due to genetic difference in the sensitivity of the combs to the comb-growth-promoting hormone. A similar conclusion is arrived at from the maintenance experiments described below.

One rather curious point of difference in behaviour between the Leghorn and Plymouth Rock combs may be mentioned here. When the latter was almost stationary at quite a small size on 0·5 mg. androsterone daily, it was very noticeable that the papillae became well developed, more so than those of the fast-growing Leghorn comb. Further, the papillae of the comb of the Leghorn described on p. 1416 underwent comparatively little development during the phase of very rapid comb growth, but grew later to the normal size. This difference during induced growth is obviously correlated with the difference shown during atrophy. The comb of the Leghorn after adult castration very soon loses its papillae, and at 10 days after operation it is usually quite smooth, as well as being very blanched. The comb of the Plymouth Rock, on the other hand, retains papillae and a red colour for much longer after adult castration.

IV. *Maintenance of full comb size.*

Brown Leghorn. An adult Brown Leghorn cock, comb 12·9 × 7·8 cm., was castrated. 10 days later, when the comb was showing easily appreciable shrinkage and loss of colour, the injection of 1 mg. of androsterone daily was started and was continued for 12 days. This treatment caused the comb to regain colour, and the papillae began to reappear. The effect on the course of regression is shown in Fig. 4. After the first 3 days the size of the comb increased steadily, and had the injections been continued the comb would presumably have been maintained at a greater size than when injections were begun, even if not at the size observed at the time of operation. This experiment therefore confirms the supposition that the full-sized Leghorn comb can be maintained by about 1 mg. androsterone daily.

Plymouth Rock. Two Plymouth Rocks were castrated when adult. 11 days later, when both combs had shrunk considerably but were about equal in size, injection of 1 mg. androsterone daily into one bird was started, whilst the other was kept as a control. Subsequently the dosage to the first bird was raised to 2·5 mg. daily. Details of the size changes in the combs are shown in Fig. 5. The dose of 1 mg. daily restored the comb to just about its previous size and would, no doubt, have maintained it at this level. Raising the dose at this point increased the size of comb slightly, but at the time of killing there were signs that the comb size was becoming stabilised at a point not far above the normal.

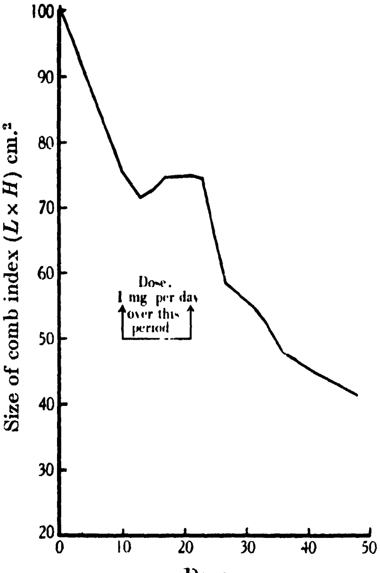


Fig. 4.

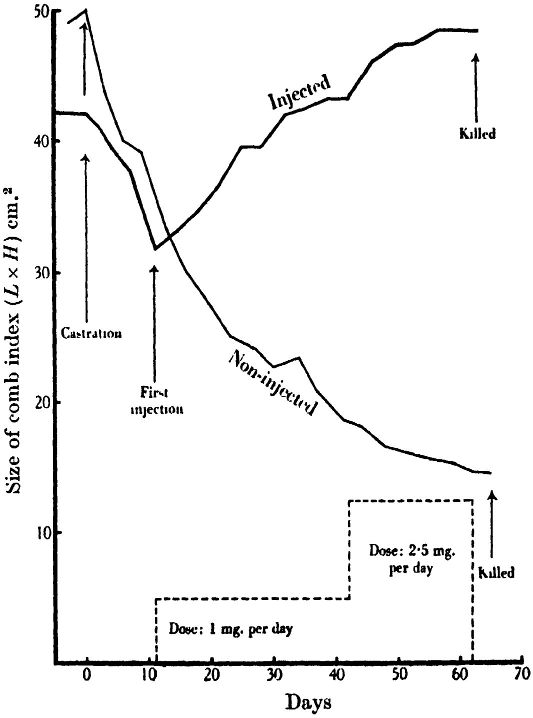


Fig. 5.

Fig. 4. Maintenance of comb size after adult castration of Brown Leghorn cock.

Fig. 5. Maintenance of comb size after adult castration of Plymouth Rock cock.

V. *Comparative sensitivity of combs of Leghorn and Plymouth Rock capons.*

The experiments recorded above show that the testes of the Plymouth Rock cock produce as much comb-growth factor as those of the Leghorn or even more, and that the lesser size of the Plymouth Rock comb is due to its lesser sensitivity. This conclusion has a bearing on a matter of practical importance, *viz.* choice of breed for capon comb assay work. White and Brown Leghorns have been used almost exclusively up to date, but Plymouth Rocks have the advantage that they can be easily caponised at a much later stage than can Leghorns. As a further check therefore on the difference in sensitivity, a group of 5 Leghorn capons and one of 5 Plymouth Rock capons were tested. The Leghorns were given 1 mg., and the Plymouth Rocks 5 mg. over 5 days, the final comb measurements being made 1 day after the last injection (Table I). This dosage was arranged in the hope of getting a good measurable response in both groups.

Greenwood *et al.* [1935] have shown that in Leghorns over this range of growth the size increase has an almost linear relation with the dosage, and it is

Table I.

	Average $L+H$ at start mm.	Average $L+H$ increase mm.
5 Leghorn capons each receiving 1 mg. over 5 days	45 + 20	11
5 Plymouth Rock capons each receiving 5 mg. over 5 days	42 + 16	10·7

unlikely that the Plymouth Rock combs exceeded the range over which the relation is linear. One must therefore conclude that to obtain a similar response in the Plymouth Rock capon demands about 5 times the dose required for the Leghorn capon. Breed thus appears to be an important variable in assay by the comb-growth method.

VI. *Histological observations on the vas deferens.*

The paper by Callow and Deanesly [1935] shows that pure androsterone is relatively inefficient in causing growth of the accessory organs of castrated mammals. It is thus important to know whether the internal accessory organs of capons show a response comparable with that of the comb or whether, like the mammalian accessory organs, they only respond to relatively high doses. In practice one is reduced to examination of the effect on the rudimentary or atrophic vas deferens of the capon. In the present series the vasa deferentia of the Leghorn recorded on p. 1416 and in Fig. 2, of the 2 Plymouth Rocks (p. 1418 and Fig. 5) and of an uninjected Leghorn capon are available for histological examination. The vas deferens of the uninjected capon, as might be expected, was greatly atrophied; in places the lumen, lined by low cuboidal cells, was scarcely patent (Plate IV, fig. 9). That of the uninjected Plymouth Rock, 65 days after adult castration, was less atrophic, but had obviously undergone extensive involution (Plate IV, fig. 11). In the injected Plymouth Rock the vas deferens was fully maintained as regards the appearance of the epithelium, and the general size, though subnormal, was much greater than in the uninjected control (Plate IV, fig. 12). A similar condition (Plate IV, fig. 10) was shown by the vas deferens of the Leghorn (EC 5). In view of the fact that the large size of the normal vas deferens must be partly due to distention with testicular products, it is likely that the vasa deferentia of these two capons were almost completely maintained and developed respectively by the administration of androsterone. In comparing these results with those on rodents, it should be remembered that very large amounts of androsterone were given over prolonged periods.

VII. *Effect of androsterone on the comb of the hen.*

It seems to be established that oestrin has only the smallest effect, if any, on the comb of the hen. The question thus arises as to what induces comb growth in the hen and in particular why does the comb increase in size and colour when the bird is in lay? The obvious reply is to suggest that in the same way as the fowl testis produces oestrin in addition to male hormone, so the ovary produces male hormone in addition to oestrin. We are not however aware of any experiments on the effect of male hormone on the comb of the hen.

Two hens were injected with androsterone. The first was a Rhode Island Red with a small, erect comb, and the second was a Brown Leghorn with a large, drooping comb. 2·5 mg. daily were given: the results are shown in Fig. 6. The enlargement produced was of quite a different order from that in the capon comb. In 3 weeks the comb of the Rhode Island Red barely doubled in $L \times H$, while the similar comb of the very refractory Plymouth Rock capon (p. 1418, and fig. 3) more than doubled in $L \times H$ in 8 days on the same dosage. The Leghorn hen's comb increased in $L \times H$ from 22·0 cm.^{2} to 45·1 cm.^{2} in 33 days. This may be contrasted with the Leghorn capon EC 8 which, on a dose of only 0·5 mg. daily, increased in $L \times H$ from 34 cm.^{2} to 63 cm.^{2} in 26 days.

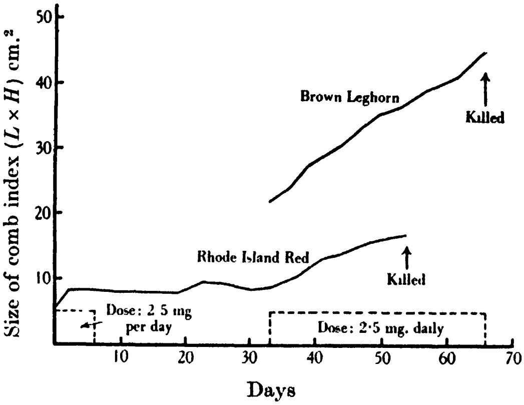


Fig. 6. Effect of androsterone on hens' combs.

VIII. *Discussion.*

It is clear from the results described above that the administration of large amounts of androsterone to capons will induce comb growth at a rate well above normal, and that crowing and "treading" occur. Further, this normal condition can be maintained by moderate daily doses. Increased efficiency is known to result from more frequent dosing [*cf.* Freud *et al.*, 1932], and moreover the comb-growth-promoting substance is probably liberated from the testis in a more effective manner than can be simulated by injection in oil. The experimental results show that daily injection of about 1 mg. of androsterone is equivalent to the comb-growth-promoting activity of the normal testis, and there is little difficulty in supposing that the testes could produce sufficient androsterone to account for the total endocrine activity as far as comb size and behaviour are concerned. It must be remembered however that there is no evidence that the testes actually do produce the particular chemical compound androsterone, which may be merely the excreted form of the testicular hormone.

The few results on the behaviour of the vasa deferentia do not allow of such definite conclusions as to the sufficiency of androsterone, and it must remain an open question whether, as in the case of rodents [Callow and Deanesly, 1935], some other substance may be involved. It should be mentioned that Callow and Parkes [1935] have shown that the plumage peculiarities of breeds such as the Sebright Bantam can only be accounted for by the assumption that the avian testis produces oestrogenic material.

A large difference was observed in the response of two different breeds of fowl to doses of androsterone. Doses of the same order are required for restoration of the comb to normal size in either Brown Leghorns or Plymouth Rocks, and the same applies to the doses necessary for maintenance. The relative sizes of the normal combs of the two breeds are however so different that the absolute increase of size of comb is several times as much for a given dose in the Leghorn as it is in the Plymouth Rock. Stated rather differently, the Leghorn comb is several times as sensitive as the Plymouth Rock comb.

It is of interest to compare the rates of comb growth obtained with these large doses given once daily with the rates obtained on similar birds under the conditions of assay employed by Greenwood *et al.* [1935]. Although a linear relation between response and dose is observed for small doses over a limited range, for larger doses (up to 8 mg. given over 5 days) the response is closely proportional to the logarithm of the dose. In the case of the Leghorn capon plotted in Fig. 2 an increase in length + height of 31 mm. occurred in 5 days with a total dose of 25 mg., and this response is of the order which would be expected on the basis of the assay results. It should be emphasised that prolonged administration of a smaller dose of androsterone will produce a comb of normal size with less total material than a brief period of heavy dosing.

As regards changes in the rate of increase on a constant dosage, Gallagher and Koch [1930] showed that with a moderate daily dosage the rate of increase was fairly constant for about a week and then fell off rapidly until the comb was approaching stability. The work recorded here shows that this kind of thing happens over a wide range of dosage, the steepness of the initial rise and the time taken for the growth curve to flatten off depend on the magnitude of the dose given, and, of course, on the breed of bird. The first Leghorn (EC 5) showed an unbroken straight line for comb growth during the period when it was getting 5 mg. daily. Such a rate of growth could obviously not have been maintained much longer, but it is probable that a very large comb would have been produced if material had been available to continue the heavy dosage indefinitely.

The relatively much lower growth rate found in hens after injection of androsterone raises an interesting problem. The strain of egg-laying might have some effect in inhibiting comb development, but actually during the period of the experiment the Rhode Island Red hen laid 1 egg only, and the Leghorn hen 2 eggs only, though the ovary was fully functional at autopsy. Three other explanations suggest themselves: (*a*) that the hen's comb is inherently less responsive to male hormone than the cock's, (*b*) that some substance in the hen, possibly oestrin, inhibits the action of androsterone on the comb, and (*c*) that androsterone is transformed in some way by the ovary. The first suggestion is difficult to reconcile with the known effects of testicular grafts on ovariectomised hens. As regards the second suggestion, the simultaneous injection of oestrin and male hormone concentrate according to Schoeller and Gehrke [1933] may actually increase the effect of male hormone on the comb. In an experiment carried out on capons we found that 1 mg. of androsterone *plus* 1 mg. of oestrone administered over 5 days gave a slightly lower response than the administration of 1 mg. of androsterone alone. This isolated result is not in accordance with the results of Schoeller and Gehrke, nor, on the other hand, was the difference sufficient to explain the results with intact hens. The third suggestion is at the moment speculative, and further investigation of the phenomenon is obviously necessary.

SUMMARY.

1. Very rapid growth of the combs of capons, with complete restoration to normal size in the course of a few weeks, may be induced by injection of crystalline androsterone in doses of the order of 2·5–5 mg. daily.

2. The comb can be maintained at the normal level by daily doses of the order of 1 mg. of androsterone. This requirement is not inconsistent with the view that secretion of androsterone accounts for the whole androkinetic activity of the testis of the cock. Observations on the restoration of the vas deferens have also been made.

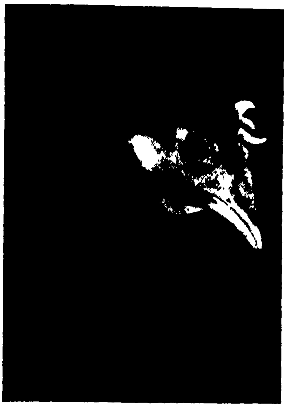


Fig. 7.

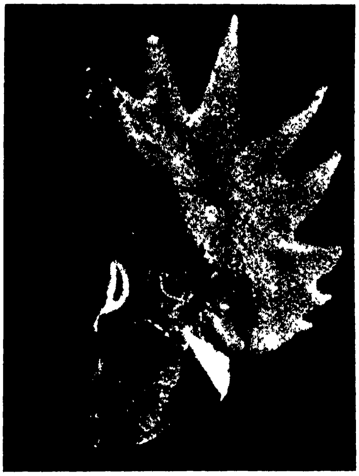


Fig. 8.

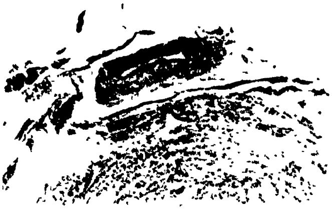
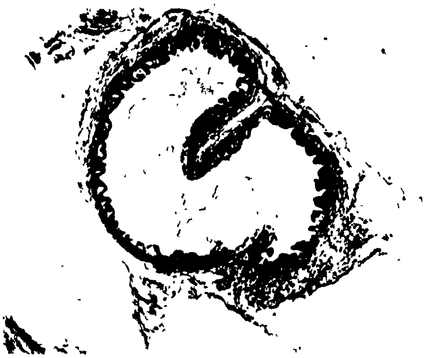


Fig. 9.



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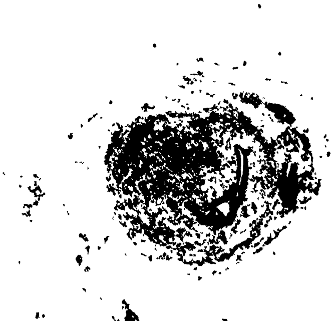

Fig. 11.

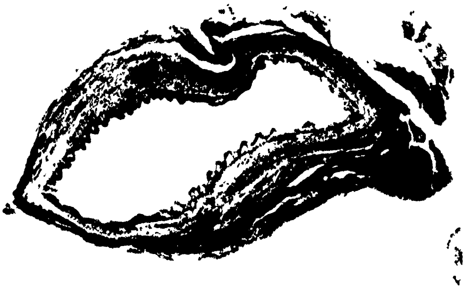

Fig. 12.

3. The comb of the Leghorn capon is several times more sensitive to androsterone than that of the Plymouth Rock capon.
4. The comb of the hen is much less responsive than that of the capon.

We are indebted to Dr A. W. Greenwood, of the Institute of Animal Genetics, Edinburgh, for two Brown Leghorn capons used in this work.

REFERENCES.

Appel (1929). *J. Morph. Physiol.* **47**, 497.
Callow and Deanesly (1935). *Biochem. J.* **29**, 1424.
—— and Parkes (1935). *J. Exp. Biol.* (in press).
Freud, Laqueur and Pompen (1932). *Endokrinol.* **10**, 1.
Gallagher and Koch (1930). *J. Pharm. Exp. Ther.* **40**, 327.
Greenwood, Blyth and Callow (1935). *Biochem. J.* **29**, 1400.
Hill and Parkes (1934). *Proc. Roy. Soc. Lond.* B, **116**, 221.
McGee, Juhn and Domm (1928). *Amer. J. Physiol.* **87**, 406.
Ruzicka, Goldberg, Meyer, Brüngger and Eichenberger (1934). *Helv. Chim. Acta*, **17**, 1395.
—— and Tschopp (1935). *Naturwiss.* **23**, 44.
Schoeller and Gehrke (1931). *Wien. Arch. inn. Med.* **21**, 329; *cf. Zahnärztl. Mitteil.* No. 13, March 29th.
—— —— (1933). *Arch. Gynäkol.* **155**, 234.

DESCRIPTION OF FIGURES IN PLATE IV.

Fig. 7. Leghorn capon EC 5 before injection.

Fig. 8. The same bird after 22 daily injections of 5 mg. androsterone.

Fig. 9. Vas deferens of uninjected Leghorn capon, showing total atrophy. × 48.

Fig. 10. Vas deferens of EC 5. Complete development of epithelium and increase in size. × 48.

Fig. 11. Vas deferens of post-pubertally castrated Plymouth Rock (EC 3), 65 days after operation. Uninjected. × 48.

Fig. 12. Vas deferens of post-pubertally castrated Plymouth Rock (EC 2), 63 days after operation. Injected as stated in Fig. 5, showing complete maintenance of epithelium, though the duct is not quite full size. × 48.

CLXXIV. EFFECT OF ANDROSTERONE AND OF MALE HORMONE CONCENTRATES ON THE ACCESSORY REPRODUCTIVE ORGANS OF CASTRATED RATS, MICE AND GUINEA-PIGS.

By ROBERT KENNETH CALLOW and RUTH DEANESLY.

From the National Institute for Medical Research, London, N.W. 3.

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I. Introduction.

The accessory reproductive glands of castrated rodents have long been recognised as suitable objects for testing the activity of male hormone preparations. A full bibliography will be found in Koch [1932] and the other literature cited below. Moore and Gallagher [1930, 2], enumerating mammalian indicators for male hormone, state that, after the spermatozoon motility test (one not in general use), the restoration of normal prostatic structure is the most sensitive test, the next being cytological restoration of the seminal vesicles. Many tests of this kind have been made, but although histological changes in the glandular epithelium have been brought about by a few days' treatment, yet the stimulation or maintenance of glands of normal adult size in groups of animals castrated for as long as 10 or 14 days has not been quantitatively demonstrated, until recently by Dingemanse *et al.* [1935]. These workers give the average seminal vesicle weights in 5 immature castrated rats injected for 24 days with a testis extract as 538 mg., approximately equivalent to the glands in a normal 175 g. rat (Fig. 3) and about two-thirds of the full size. Now that androsterone, the male hormone first extracted from urine, can be obtained artificially in larger quantities, by the method of Ruzicka *et al.* [1934, 2; Ruzicka, 1935], it has been possible to make an extended quantitative investigation of the responses in weight of the prostate and seminal vesicles. For comparative purposes, a number of experiments have also been made with several urine concentrates.

The two types of experiment which have been carried out are: (1) maintenance of normal size glands in rats and mice castrated as adults, and (2) production of growth in the glands of prepubertally castrated rats, mice and guinea-pigs.

An experiment of the first type was reported by Moore and collaborators [1930], who state that they maintained in normal condition the accessory glands of two adult castrated rats for 20 days by injections of crude male hormone begun on the day following castration; unfortunately no figures are given for either the weight of the rats or the weight of the glands. Hansen [1933] also carried out maintenance experiments on rats for 10-day periods but the accessory glands were not weighed, only examined histologically. Martins [1930] carried out similar partial maintenance experiments on mice of about 18 g. weight, using a length-breadth index of seminal vesicle size.

Many experiments of the second type have been reported on rats [Moore and collaborators, 1930; Freud *et al.*, 1930; 1932; Korenchevsky and collaborators, 1932–1935] and others have been carried out on mice [Voss and Loewe, 1931].

The actual increases in prostate and seminal vesicle weights (particularly the latter) caused in these prepubertally castrated rats and mice have in most cases been so small as to be almost negligible (a point emphasised by Voss and Loewe) and consequently the majority of these workers have relied on histological changes as an index of the activity of their extracts. Korenchevsky and his co-workers alone have attempted to base a quantitative method of assay on the increase in weight of the atrophied glands.

Freud and his collaborators have tried to obtain improved results in this type of experiment by combinations of oestrin with male hormone urine concentrates. They have also tested various oestrin derivatives [David *et al.*, 1934]. For assaying purposes these workers rely on the capon comb test, but Freud [1933] notes that in the purification of extracts male hormone activity could be lost as regards rats while being retained as regards capons [*cf.* also Laqueur, de Fremery *et al.*, 1931].

The present experiments were designed not only to test the biological activity of pure androsterone on rodents but also to compare it with the activity of crude urine extracts, since a significant difference between the two might imply the natural occurrence of more than one active substance.

II. MATERIAL.

Note on nomenclature. The term androsterone is applied to the crystalline compound first isolated by Butenandt from urine and prepared artificially from cholesterol by Ruzicka and his co-workers. The term "male hormone" is applied generally to this substance and to concentrates from natural sources without implying that they are necessarily identical. It seems desirable to avoid such terms as "testicular hormone from urinary sources" in the present state of knowledge.

Crystalline androsterone. The method described by Ruzicka *et al.* [1934, 2] for the preparation of androsterone from cholesterol was followed closely except in the preparation of *epi*cholestanol and in the isolation of androsterone in the final stage. The reduction of cholestanone was carried out in quantities of 100 g. dissolved in 3 litres of amyl ether. To this were added 100 ml. of concentrated hydrochloric acid and platinum black prepared by Vavon's method [1914] from 35 g. of platinum chloride. Absorption of hydrogen was complete in 4 hours with fresh catalyst. After filtering the hot solution from the catalyst, the *epi*cholestanol was allowed to crystallise out and the mother-liquors were used for a second reduction. The addition of fresh catalyst was necessary after four reductions. A yield of 397 g. of recrystallised *epi*cholestanol, M.P. 180–183°, was obtained from 786 g. of cholestanone in one series of reductions.

Some attempt was made to modify the conditions of the oxidation of *epi*cholestanyl acetate, the most wasteful stage in the preparation. Oxidation at a higher temperature (102–105°) for a shorter time yielded however a ketonic fraction containing much oily material. The isolation of the ketonic fraction was carried out with the help of the new reagent trimethylaminoacetohydrazide chloride, $Cl.Me_3N.CH_2.CO.NHNH_2$ [Girard and Sandulesco, 1934]. The neutral fraction of the oxidation product from one batch (80 g.) of *epi*cholestanyl acetate, freed from the bulk of unchanged material and methylheptanone, was evaporated to dryness and dissolved in 25 ml. of glacial acetic acid. To this solution was added a solution of 10 g. of Girard's reagent in 25 ml. of acetic acid and the mixture was warmed gently for a short time. The mixture was then poured into water and oily material extracted with ether. The aqueous layer, containing the ketonic fraction as a water-soluble hydrazone, was then warmed on the water-bath with a slight excess of dilute sulphuric acid; the material which separated was extracted with ether, and the extract evaporated. The crude acetate thus obtained was hydrolysed with alcoholic sodium hydroxide and yielded 1·4 g. of crude ketone, M.P. about 120°, which was recrystallised from ethyl acetate. Crude androsterone (0·4 g., M.P. 168–176°) separated, mixed with flocculent material. Recrystallisation from ethyl acetate, a good, though rather wasteful, solvent for the purpose, yielded pure androsterone, M.P. 184–185°, $[\alpha]_D^{20^\circ}+93·6^\circ$, $[\alpha]_{5461}^{20^\circ}+115·8^\circ$ in absolute alcohol. About 3 g. of androsterone were obtained from 3 kg. of cholesterol by this method. Under good conditions the over-all yield was about 0·2 % of the theoretical.

Fractional crystallisation of the product from the mother-liquors and the flocculent material accompanying the crude androsterone yielded a *ketone*, M.P. 166–167°, from aqueous alcohol, $[\alpha]_D^{20^\circ}+20^\circ$, $[\alpha]_{5461}^{20^\circ}+22^\circ$ in absolute alcohol. It showed no selective absorption in the ultra-violet in 0·1 % solution. This material did not induce measurable comb growth in capons in a total dose of 10 mg. over 5 days. Its composition has not yet been further investigated, but it seems likely that it is a product of incomplete removal of the sterol side-chain, possibly *epiallo*pregnanolone.

We are indebted to Mr A. L. Bacharach of Glaxo Laboratories for the supply on special terms of the cholesterol used in this preparation.

In addition to the material prepared in the laboratory, about 1·4 g. of androsterone were placed at our disposal by Messrs Ciba of Basle, to whom our very best thanks are due for this most generous assistance. A supply of androsterone from natural sources (50 mg. of crystalline "Proviron") was very kindly made available by Prof. Schoeller, Schering Kahlbaum A.G., Berlin.

Solutions of androsterone in arachis oil for injection were prepared by mixing at 100–150°. Greenwood *et al.* [1935] have shown that androsterone is stable under these conditions.

Male hormone concentrates.

(*a*) 2300 capon units of "Proviron" urine concentrate were given by Prof. Schoeller.

(*b*) 1000 capon units of "Enarmon" urine concentrate were given to us by Dr Itoh, Teikokusha Institute, Kawasaki, whom we also take this opportunity of thanking.

(*c*) A small supply (240 capon units) of "Hombreol" Organon was purchased, but only two experiments were possible with this material because of its high price.

iso*Androsterone*. 3-Hydroxy-17-keto-aetio*allo*cholane, isomeric with androsterone, referred to as *iso*androsterone for convenience, was prepared from cholestanyl acetate as described by Ruzicka *et al.* [1934, 1], but was isolated with the help of Girard's reagent.

Oestrin was administered in the form of oestrone (ketohydroxyoestrin) or, in earlier work, oestriol (trihydroxyoestrin) for which we are indebted to the British Drug Houses, Ltd.

Comb growth promoting activity of the materials. As a basis for comparison of the results obtained on rodents, the various preparations were first tested on Brown Leghorn capons belonging to Dr Greenwood's stock, but kept in London. The assay of pure androsterone differs slightly from that carried out in Edinburgh by Dr Greenwood [Greenwood *et al.*, 1935], probably because of differences in environmental factors, but the assays in London were all carried out under identical conditions, and should thus be comparable with each other, the only essential point. The results of these assays are given in Table I.

Table I. *Assays of male hormone preparations on capon combs.*

Material	Daily dose for 5 days	Av. comb increase $L+H$ mm.	Wt. or volume of capon unit (approx.)	Capon units per mg. or per ml.
Androsterone (Ciba)	0·2 mg.	10·9	100γ	10 per mg.
Androsterone (present paper)	0·2 mg.	9·3		
*iso*Androsterone	1·4 mg.	12·0	600γ	1·6 per mg.
"Enarmon"	0·2 ml.	10·2	0·1 ml.	10 per ml.
"Proviron"	0·1 ml.	9·7	0·05 ml.	20 per ml.
"Hombreol"	0·1 ml.	11·1	0·045 ml.	22 per ml.

Each assay was carried out on a group of 5 capons, and the capon unit is defined as that amount of preparation which, injected daily for 5 days, produces an average increase in $L+H$ of 5 mm. one day after the last injection. For purposes of calculation, it is assumed that the dose/response relation is linear up to a $L+H$ increase of 12 mm., a quite warrantable assumption [Greenwood *et al.*, 1935]. The dosage in each case was an amount expected to give about 10 mm. increase in $L+H$ in the 5 days, *i.e.* a good accurately measurable response.

The weight of the capon unit of crystalline androsterone agrees closely with that obtained by Ruzicka *et al.* [1934, 2], using a slightly different method of assay, involving however a very similar amount of growth. In the case of *iso*androsterone, close agreement is also seen with Ruzicka's assays, in which it was found that the isomeride had about one-seventh the activity of the hormone; the above assays make the ratio 1 : 6. For comparative purposes the activity of crystalline androsterone is taken as 10 capon units per mg. Only two samples of androsterone were assayed in London, but a series of assays carried out by Dr Greenwood failed to show any significant difference in activity between androsterone "Ciba", androsterone prepared in this laboratory and androsterone "Proviron" from urine.

The "Enarmon" preparation, a urine concentrate in oil, was labelled as 10 capon units per ml., no information being available as to the method of testing. The above assay entirely agrees with this definition of its strength. "Enarmon" was also slightly oestrogenic. 1 ml. of "Enarmon" oil produced cornification in an ovariectomised rat, and 0·1 ml. oil injected into each of three ovariectomised mice gave a positive response in two.

The batch of "Proviron" urine concentrate used in this test had been in the laboratory for nearly 2 years and was labelled as 25 capon units per ml. (for method of assay, see Schoeller and Gehrke [1931]). Actually, it assayed at almost exactly 20 units per ml. by our method. The difference, if significant, may be due to difference in assay method or deterioration of the preparation.

The "Hombreol" urine concentrate was labelled as 20 units per ml. (for method of assay, see Freud *et al.* [1932]). Our assay, recorded above, agrees substantially with this description; the difference is doubtfully significant.

The remarkable thing about these assays is the close agreement between the standardisations of the four different preparations carried out in as many different countries. It is doubtful if any of the differences between the observed and labelled activities are significant: there appears to be greater unanimity as to what constitutes a capon unit than might have been expected. In view of these findings it is rather surprising that the androsterone tests carried out in Edinburgh [Greenwood *et al.*, 1935] should have given uniformly lower values, especially since the birds were from the same stock as those used in London.

III. Description of prostate and seminal vesicles; methods of dissection.

(*a*) *The rat.* The rat prostate has been frequently described; references will be found in Walker [1910]. It is illustrated in Figs. 1 *a* and 1 *b*, which show the terminology adopted in this work. The gland consists essentially of three pairs of lobes:

(1) Cranial lobes or coagulating glands attached to the length of the seminal vesicles. These are termed the anterior lobes by Moore, Price and Gallagher [1930], as were similar lobes in the mouse prostate by Deanesly and Parkes [1933]; but since Walker [1910] uses the term in another sense it seems better to discard it.

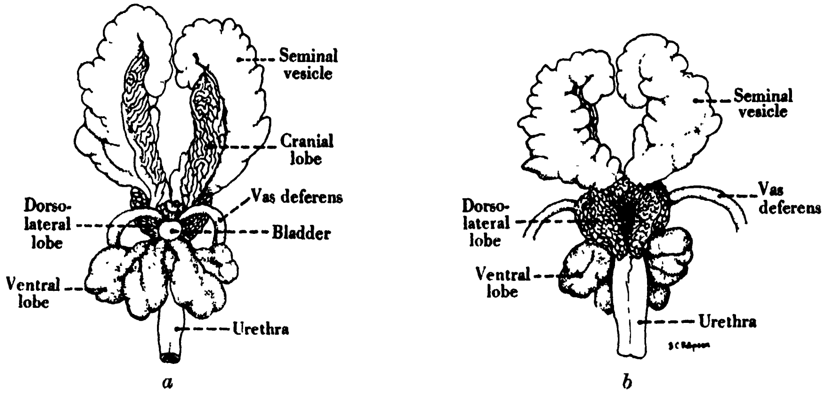

Fig. 1. Prostate and seminal vesicles of the rat. *a*, ventral view; *b*, dorsal view. The ventral lobes have been drawn back to expose the rest of the glands.

(2) Dorso-lateral lobes lying round the base of the seminal vesicles and almost encircling the urethra. Moore, Price and Gallagher [1930] call these the middle lobes, whereas Walker calls them the posterior lobes.

(3) Ventral lobes attached to the caudal end of the prostate only by their ducts. These are the posterior lobes of Moore, Price and Gallagher [1930] and the anterior lobes of Walker [1910].

In addition to these lobes there is a pair of small ventral ampullary glands at the point of entry of the vasa deferentia, which for weighing purposes may be included with (2).

The bulk of the rat prostate is made up of the dorso-lateral and ventral lobes, which are much better developed than the corresponding structures in the mouse. Since these react more readily to male hormone than the cranial lobes or the seminal vesicles, the rat is a more satisfactory test animal for small doses than the mouse.

The rat seminal vesicles are shown in Figs. 1 *a* and 1 *b* and require no description here.

The dissection and weighing of the accessory glands were carried out as follows: the entire reproductive tract was dissected free of fat, fixed in Bouin's fluid and then transferred to 70 % alcohol. At this stage the cranial prostate lobes were dissected off the seminal vesicles and the latter freed from the dorso-lateral lobes and removed for separate weighing. Next, the ventral and dorso-lateral lobes were freed from the urethral musculature by dissection from the caudal region and the vasa deferentia were cut off at their points of entry. After removal of the bladder and of any remaining connective tissue, the dissected prostate consisted almost entirely of glandular substance. In the larger prostates the three pairs of lobes were weighed separately but this was not done for the smaller glands. Dissection was carried out as rigorously as possible, but naturally prostates of 15–40 mg. were more difficult to dissect cleanly and accurately than the larger glands. All glands were thoroughly drained before weighing. No glandular tissue from inside the urethral muscle was included with the prostate, since it was desirable to simplify and standardise the dissection as far as possible.

(*b*) *The mouse.* The mouse seminal vesicles and prostate have already been described [Deanesly and Parkes, 1933]. The method of dissection used in this work was the same as before; the seminal vesicles, with the cranial (anterior) lobes of the prostate attached to them, were cut off after fixation at the level of the entry of the vasa deferentia and weighed together. The mouse prostate, particularly when atrophied, is unsuitable for clean dissection and weighing.

(*c*) *The guinea-pig.* The guinea-pig prostate has been described by Walker [1910] and Engle [1926]. Although consisting of the same three pairs of lobes it is less compact and well-developed than that of the rat and accordingly less suitable for quantitative work with male hormone, since its sensitivity is much the same as that of the rat.

The seminal vesicles of the guinea-pig are elongated tubular structures which regress after castration more slowly than those of the rat or mouse. They were dissected out from the prostate after fixation and weighed separately.

IV. GROWTH OF THE PROSTATE AND SEMINAL VESICLES OF THE RAT.

The growth and post-castration atrophy of the accessory reproductive glands of the normal mouse have already been dealt with [Deanesly and Parkes, 1933], and the growth of the seminal vesicles of the guinea-pig will be described elsewhere [Deanesly and Rowlands]. As a control on the experiments in Sections V and VI however it was necessary to make an extensive study of the growth of both the prostate and seminal vesicles of the rat. All animals used were albino and belonged to the Institute's stocks.

Growth of the prostate and seminal vesicles. The weights of the prostate and seminal vesicles were obtained in 79 normal rats, ranging in body weight from 42 g. to 250 g. The gland weight, plotted against body weight, is shown in Figs. 2 and 3, and the curves drawn through the same points are shown in Figs. 4 and 5 as a standard against which the experimental results described in Section V can be compared. In order to facilitate comparison, the growth curve for the seminal vesicles has been superimposed as an interrupted line on the prostate diagram and *vice versa.*

Although the figures show considerable variations in gland size in relation to body weight, especially in the smaller rats, the main trend of growth is quite clear. In both prostate and seminal vesicles little appreciable enlargement occurs before the body weight is 100 g. In this slight early growth the seminal vesicles, which start very much below the prostate, lag behind still farther, so that at 90 g. body weight the prostate averages about 80 mg. while the seminal vesicles are only about 20 mg. Above 100 g. body weight both the glands enlarge very rapidly and tend to approximate in size. At 150 g. body weight the prostate is rather above and the seminal vesicles rather below 300 mg. At 180 g. body weight both are about the same at 500 mg., while soon after 200 g. body weight the glands attain what appears to be their full weight of 700–800 mg. each. Above a body weight of 250 or 275 g. the prostate and seminal vesicles of the rat do not seem to show any regular increase in size although both may occasionally reach a weight of 1 g. in large rats. In some large animals however, presumably old ones, both seminal vesicles and prostate have obviously regressed. The initial lag in the growth of the seminal vesicles suggests that they have a threshold requirement different in kind or degree from that of the prostate.

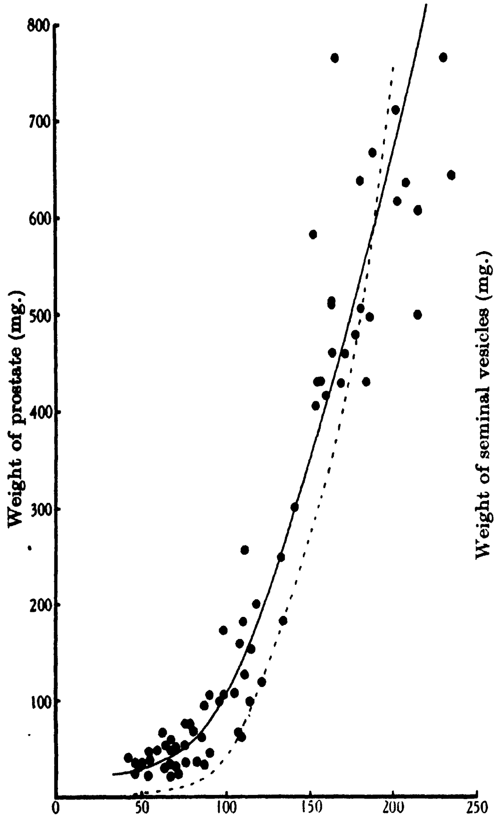

Fig. 2.

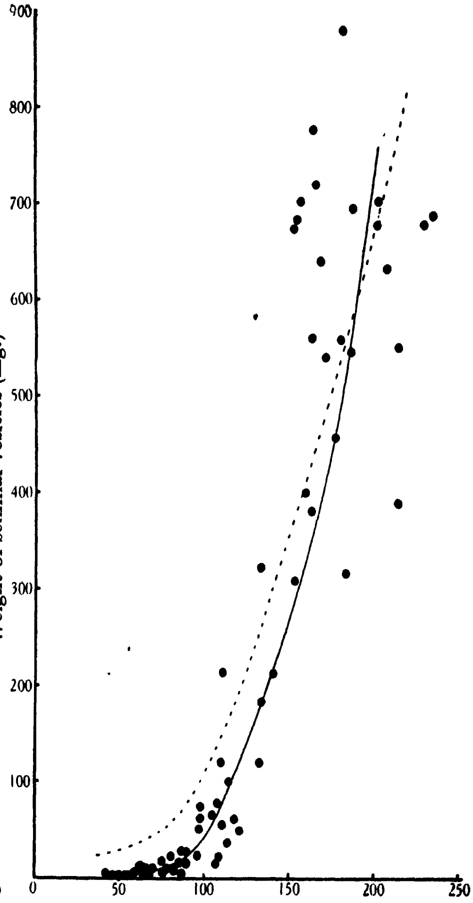

Fig. 3.

Fig. 2. —— Growth of prostate in the normal rat; ---- growth of seminal vesicles for comparison.

Fig. 3. —— Growth of seminal vesicles in the normal rat; ---- growth of prostate for comparison.

V. Maintenance of the prostate and seminal vesicles of the rat after postpubertal castration.

Previous workers have shown that in the adult rat, as in the mouse, considerable atrophy of the accessory glands takes place in the first fortnight after castration. Certain "maintenance" experiments have been carried out in which attempts have been made to replace testicular activity in sexually mature rats by injections begun immediately after castration. Since some body growth takes place in the 14 days' course of the experiments, complete maintenance of the accessory glands in their normal relation to body size implies not merely maintaining the size of the glands at the time of castration but actually inducing normal growth.

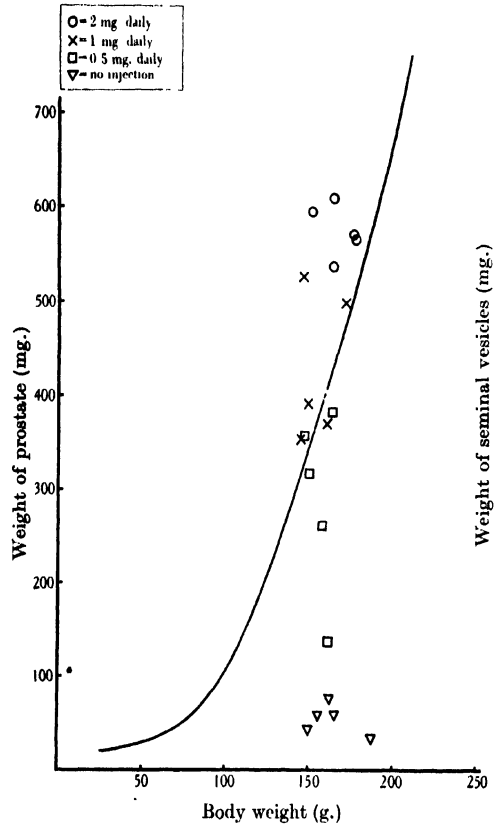

Fig. 4.

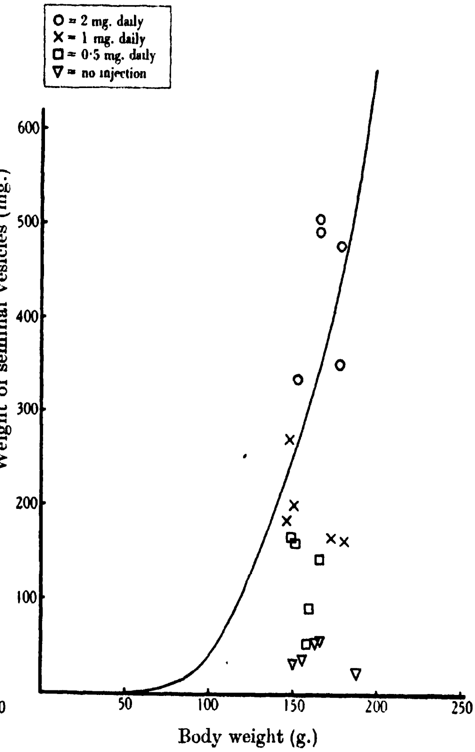

Fig. 5.

Fig. 4. Maintenance of prostate in postpubertally castrated rats by injection of androsterone.

Fig. 5. Maintenance of seminal vesicles in postpubertally castrated rats by injection of androsterone.

Three groups of five rats each of 130–155 g. body weight were castrated and injected with androsterone in arachis oil for 14 consecutive days and then killed 24 hours after the last injection. Rats of the first group were given 0·5 mg. daily, those of the second 1 mg. daily and those of the third 2 mg. daily. The weights of the prostate and seminal vesicles at autopsy are shown in Figs. 4 and 5 and Table II in relation to the normal growth curve and to their weights in uninjected normal and castrated groups.

It will be seen that:

(*a*) The prostates of all the rats receiving 1 or 2 mg. daily and of 4 of those receiving 0·5 mg. are substantially normal in size. Considered as groups, those receiving 2 mg. are rather above normal size, those receiving 1 mg. (slightly smaller animals) are almost normal, whilst those receiving 0·5 mg. are below normal (Table II).

Table II. *Growth and maintenance of rat accessory glands after postpubertal castration.*

No. of rats	Condition	Daily injection of androsterone mg.	Av. body wt. at castration g.	Av. body wt. at autopsy g.	Av. prostate wt. mg.	Av. seminal vesicles wt. mg.
5	Normal	—	—	139	283	230
5	Normal	—	—	162	511	474
5	Normal	—	—	198	624	650
5	Castrated	3	199	212	867	665
1	Castrated	—	220	205	128	168
5	Castrated	2	138	167	577	431
5	Castrated	1	138	155	429	204
5	Castrated	0·5	141	157	291	122
5	Castrated	—	138	165	55	51

(*b*) The seminal vesicles have been less well maintained. All rats receiving 2 mg., 3 of those receiving 1 mg., and 2 of those receiving 0·5 mg. have seminal vesicles approximately normal in size, but the average seminal vesicle weight in all groups is below that of the controls, although the difference is probably not significant for the group receiving 2 mg. daily.

It is apparent that while 1 mg. of androsterone daily will maintain the prostate in rats of this size after removal of the testes, about twice as much is required to maintain the seminal vesicles. This obvious difference in threshold requirement (noted in earlier qualitative experiments by Moore and Gallagher, [1930, 2; Hansen, 1933]) can be associated with the difference in size in the early stages of growth.

Hansen [1933] estimates the maintenance requirements of seminal vesicles of adult rats at six or seven of Gallagher and Koch's capon units of testicular extract [Koch, 1932], whereas two such capon units are ample for the prostate.

In order to ascertain whether the larger glands of larger rats required a correspondingly increased amount of hormone for maintenance, a group of 5 rats 185–230 g. in weight were castrated and given 3 mg. daily for 14 days and then killed. The seminal vesicles averaged 665 mg., and, allowing for normal variation, were apparently fully maintained (Table II). The prostates averaged 867 mg. and were, if anything, exceptionally large. Only one uninjected castrated control was used for this group, since the previous experiments had shown that the glands of rats receiving injections should be compared with normal ones rather than with those of castrates.

VI. STIMULATION OF THE PROSTATE AND SEMINAL VESICLES OF THE RAT AFTER PREPUBERTAL CASTRATION.

(*a*) *Body weight at castration; size of prostate and seminal vesicles.* In the experiments recorded below an attempt was made to stimulate rudimentary accessory glands with male hormone preparations. Rats were castrated at 35–70 g. weight, the majority at 40–50 g., before prostate or seminal vesicles had grown appreciably. In ten normal rats, 51–67 g. in weight, the average prostate size was 34 mg. and that of the seminal vesicles 4 mg. A control series of 16 uninjected rats, castrated at 35–70 g. and killed 16–68 days afterwards (with the same body weight range as the experimental animals), had prostates averaging

16 mg. (9–25 mg.) and seminal vesicles of 4 mg., the latter unaffected in weight by castration. These rats obviously provide a very low starting point for growth experiments. The figures given by Freud [1933] and David *et al.* [1934] for rats of this size and smaller are of the same order, but are rather lower for the prostate and higher for the seminal vesicles. Apparently in their method the whole prostate is not weighed together; the seminal vesicles are weighed (fresh) with a connecting bridge of prostatic tissue, and of the rest of the prostate only the weight of the "anterior" lobes is given. The peri-urethral tissue is weighed separately. Exact comparisons with the present experiments cannot therefore be made. To the differences in presenting the data must be added the fact that the Amsterdam workers in earlier experiments inject the young rats only 3 days after castration. More recently, Dingemanse *et al.* [1935] give the average weight of the prostate and seminal vesicles in 5 prepubertally castrated rats as 8 mg. and 6 mg. respectively.

The figures (50–70 mg.) given by Korenchevsky *et al.* [1932, 1934, 1, 2], although relating to Bouin-fixed prostate and seminal vesicles from prepubertally castrated rats, are so much higher than our own that they imply a difference of technique.

(*b*) *Method of testing.* Rats were injected, generally in groups of 5, beginning 20–90 days after castration. No attempt was made to standardise either the time after castration or the body weight of the animals at the time of injection, since the series of control rats showed that these factors did not influence appreciably the size of the rudiment. Most of the rats were injected at 100–220 g. weight [*cf.* Dingemanse *et al.*, 1935]. The dose is generally expressed as the total dose per rat.

Different preparations and amounts of male hormone were injected into groups of 5 prepubertally castrated rats, for a period of 10 days unless otherwise stated, after which the animals were killed and the growth and anatomical changes in the accessory glands were noted.

(*c*) *Administration of androsterone.* Ten groups of rats were given androsterone alone; the details are set out in Table III.

Table III. *Effect of androsterone on the immature castrated rat.*

No. of exp.	Days injected	Total dose per rat mg.	Medium and amount daily (ml.)	Average weight of Prostate mg.	 Seminal vesicles mg.
34	5	3·5	2·5 10% alcohol	37	9
33	10	3·5	1·0 "	67	11
44	10	4·0	2·0 "	57	8
42	10	20·0	0·1 70% "	170	56
38	10	3·5	0·1 arachis oil	44	9
56	10	6·0	0·1 "	64	14
45	10	10·0	0·1 "	112	32
57	10	15·0	0·1 "	129	17
46	10	20·0	0·2 "	129	26
63	28	28·0	0·1 "	394	46

The average weights of prostate and seminal vesicles in control rats are 16 and 4 mg. respectively. There are 5 rats in each experiment except No. 56 where there were 6.

It is immediately obvious that androsterone does not produce such striking growth in the accessory glands of the castrated rat as can be produced by oestrin in the uterus of the ovariectomised female. In the case of the seminal vesicles particularly, the growth is trifling compared with that of the normal gland. The following observations may be made with reference to these experiments.

(i) From experiments 33 and 34, it is clear that a small dose is more effective when spread over 10 days than over 5 days. From experiments 46 and 63, it also seems that the efficiency of large doses on the prostate can be greatly increased by extending the time over which administration is spread.

(ii) A comparison of the five groups receiving graduated amounts of androsterone in arachis oil over 10 days (Exps. 38, 56, 45, 57, 46, Fig. 6) suggests that the maximum growth in that period will produce a prostate of only about 130 mg. Certainly, very little is gained by increasing the total dose from 10 mg. to 20 mg. It is clear however that a prostate can actually grow to a greater weight than 130 mg. in 10 days. Thus in experiments with immature 40–50 g. normal rats, prostates averaging nearly 200 mg. were produced as the result of testicular stimulation for 10 days with prolan injections. In other experiments with androsterone (*e.g.* Exp. 42), prostates of more than 130 mg. were produced. The response of the seminal vesicles was poor in all the five groups referred to above, a total dose of 10 mg. androsterone actually giving the greatest effect.

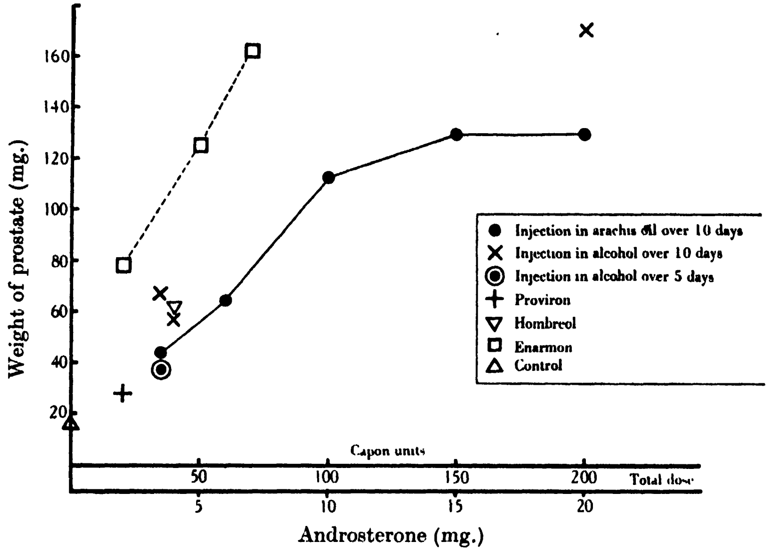

Fig. 6. Growth of prostate in prepubertally castrated rats by injection of male hormone.

(iii) It is possible that the relative ineffectiveness of the large doses may have been due, at least in part, to inefficient absorption of the material from oil. The old observation that oil is a bad medium for subcutaneous injection of rodents is amply confirmed by these experiments. Thus 3·5 mg. given as a colloidal solution in aqueous alcohol caused apparently twice as much growth in the prostate as the same amount of androsterone given in 0·1 ml. of oil daily. Similarly a total dose of 20 mg. in 70 % alcohol produced a much better effect than a total of 20 mg. in 0·2 ml. of oil daily. The increased growth of the seminal vesicles was particularly noticeable in Exp. 42. Both prostate and seminal vesicles in this group of rats show a greater approach to their maximum 10-day growth. It is difficult to ascribe these differences to any factor other than better absorption from the aqueous-alcoholic solutions. Unfortunately, only very dilute colloidal solutions in 10 % alcohol can be made, and the stronger alcoholic solutions caused bad local reactions. It should be noted that even where intramuscular injection of the oil is possible, as in the capon, aqueous media appear to be more efficient.

(iv) Exp. 63, in which the rats were injected with 1 mg. androsterone daily for 28 consecutive days, gave a very interesting result. The prostate size was nearly 400 mg., more than three times as large as that produced by a daily dose of 1·5 mg. for 10 days, but the seminal vesicles, as in all the experiments recorded in Table III, were small, weighing 46 mg., and consequently the relationship between the size of the two glands was quite abnormal for that stage of prostate development [*cf*. Korenchevsky, 1935]. In a normal rat with a 400 mg. prostate the seminal vesicles would be very similar to it in size.

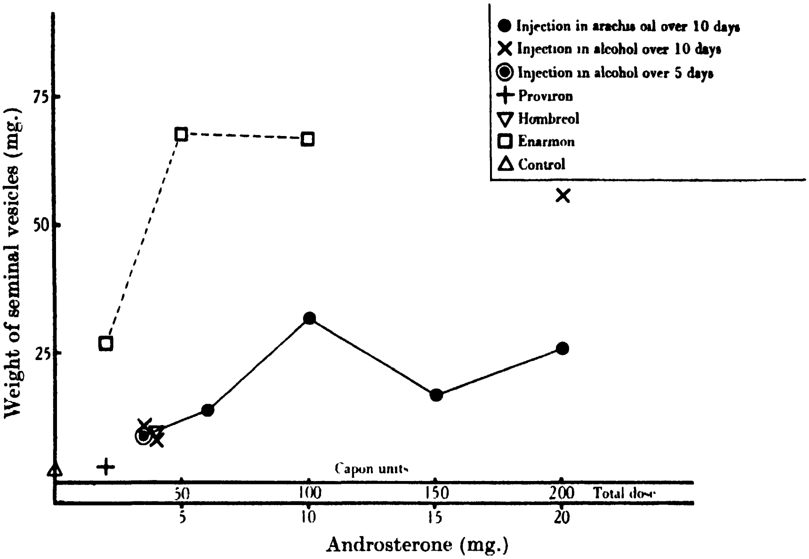

Fig. 7. Growth of seminal vesicles in prepubertally castrated rats by injection of male hormone.

This suggests that although androsterone in sufficient doses will maintain the seminal vesicles in a normal condition after castration and will also grow them from a rudimentary size up to a stage containing secretion, yet it is not as active on these glands as male hormone preparations from testis [Dingemanse *et al.*, 1935] or urine (Table IV). It is noteworthy however that Moore, Hughes and Gallagher [1930], who injected immature castrated rats for as much as 20 or 40 days with testis extract, never produced seminal vesicles of anything approaching normal adult size. This of course may have been due to absorption difficulties.

(*d*) *Administration of male hormone urine concentrates*. Five experiments were carried out, one each with "Hombreol" and "Proviron", and three with "Enarmon". The details are given in Table IV and Figs. 6 and 7. The results relating to both prostate and seminal vesicles are quite clear. Assuming 10 capon units per mg. of androsterone, Fig. 6 shows that a capon unit of either "Proviron" or "Hombreol" has about the same effect on the prostate as a capon unit of crystalline androsterone. The same conclusion may be drawn from Fig. 7 for the seminal vesicles. There is thus presumptive evidence that the only active principle of the two concentrates is androsterone. The activity of "Enarmon"

Table IV. *Effect of male hormone oil concentrates on the immature castrated rat.*

						Average weight	
No. of exp.	No. of rats	Male hormone	Capon units per ml.	Total dose per rat in C.U.	Days injected	Prostate mg.	Seminal vesicles mg.
52	5	"Proviron" (1933)	20	20	10	28	3
51	5	"Hombreol"	22	40	9	62	10
37	3	"Enarmon"	10	20	10	78	27
47	5	"Enarmon"	10	50	10	125	68
55	4	"Enarmon"	10	70	10	162	67

per capon unit however is more than twice as great as that of crystalline androsterone, "Hombreol" or "Proviron". The effect on the size of the seminal vesicles is more than 4 times as great. This clearly indicates that the comb growth promoting principle in "Enarmon" is not ordinary androsterone, or else that one or more supplementary substances with little or no effect on comb growth, but active on the accessory glands of rats, are present in addition to androsterone. In view of the work of Freud [1933] and David *et al.* [1934] confirmed by Korenchevsky and Dennison [1934, 1, 2], an obvious suggestion seemed to be that it was the small amount of oestrogenic substance present in "Enarmon" which was causing the difference in response. On the other hand, the fact that the prostate, which is much less affected by oestrone over short periods than the seminal vesicles, also showed the increased response, made this doubtful. The possibility was investigated by experiments on the effects of oestrone alone and of androsterone *plus* oestrone.

(*e*) *Administration of oestrone alone.* Two experiments were carried out with oestrone. The first group of rats was given 0·1 mg. oestrone each over 10 days, and the second 1·0 mg. The results in the two groups are identical and may be considered as representing the maximum development of rudimentary male accessory glands on oestrone alone in 10 days. The prostates showed some enlargement, possibly up to 50 %, while the seminal vesicles showed an increase to about 3 times the control weight (Table V). Allowing for the differences in the control weights and the duration of injection, these results are entirely similar to those obtained by David *et al.* [1934] and Korenchevsky and Dennison [1934, 1] except that the former obtained a rather greater effect on the prostate.

Table V. *Effect of oestrone alone and of androsterone* plus *oestrone on groups of immature castrated rats.*

	Total dose per rat			Average weight	
No. of exp.	Oestrone mg.	Androsterone mg.	Medium and amount daily (ml.)	Prostate mg.	Seminal vesicles mg.
40	0·1	—	0·1 olive oil	26	16
60	1·0	—	0·1 arachis oil	24	18
53	0·1	5	0·1 „	42	15
61	0·025	10	0·1 „	87	23
62	1·0	10	0·1 „	90	32
41	0·1	20	0·1 arachis oil + 0·1 70 % alcohol	175	73

5 rats in each experiment.

(*f*) *Administration of androsterone* plus *oestrone.* Four experiments were carried out on the simultaneous administration of the two hormones. Amounts

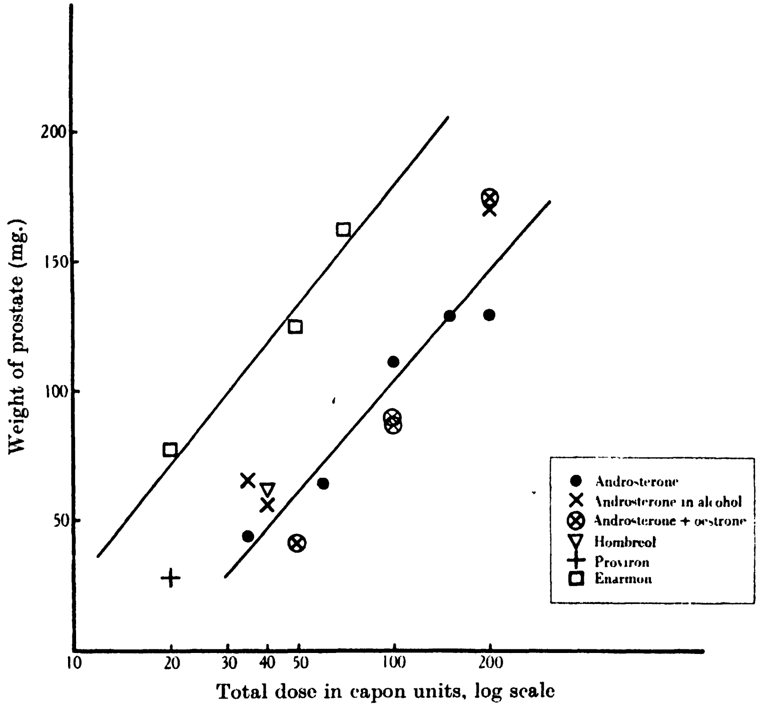

Fig. 8. Relation between growth of prostate and log dose.

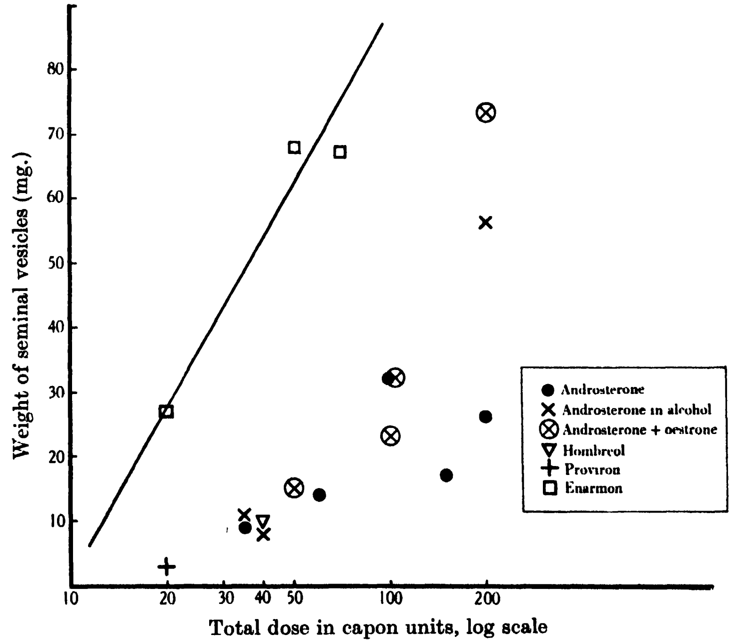

Fig. 9. Relation between growth of seminal vesicles and log dose.

of oestrone varying from 25γ (250 international units) to 1 mg. were given over 10 days; the amount of androsterone varied from 5 mg. to 20 mg. From the results in Table V it will be seen that in 3 of the 4 experiments no increase as compared with the androsterone series in Table III was evident in either seminal vesicles or prostate. In Exp. 41 the maximum dose of androsterone, *plus* 100γ oestrone, produced a prostate almost identical in size with that produced by the same dose of androsterone alone (Table III). The combined dose produced seminal vesicles slightly larger than the same dose of androsterone alone, but even so, 200 capon units of androsterone *plus* oestrone produced seminal vesicles hardly larger than those resulting from 50 capon units of "Enarmon". These results show that, under the conditions of the experiments, there is no synergistic action between androsterone and oestrone. At most there is a summation of the two different responses, the size increase of the seminal vesicles with oestrone being more marked than that of the prostate. These experiments lend no support to the idea that the extraordinary efficiency of "Enarmon" per capon unit can be due to its slight oestrin content. In Figs. 8 and 9 the responses of the prostate and seminal vesicles, respectively, have been plotted against the logarithm of the dose in terms of capon units, on the supposition that the logarithmic relation might be followed. It is actually found that in the case of the prostate (Fig. 8) the points fall roughly on two lines. There is no clear distinction between the points corresponding to androsterone, whether in oil or alcohol, androsterone *plus* oestrone, "Hombreol" or "Proviron". The results with "Enarmon" however fall quite outside the range of the others on a separate line corresponding to an activity per capon unit about three times that of the other substances. In the case of the seminal vesicles (Fig. 9) the inherent variability of the test object causes difficulty, but it is possible to suggest a line passing through the "Enarmon" points corresponding to about four times the activity per capon unit of the other materials.

(*g*) *Administration of* iso*androsterone*. Five immature castrated rats each received a total dose of 24·5 mg. of *iso*androsterone given in 0·2 ml. arachis oil daily for 10 days. This was equivalent in capon units to 4·0 mg. of androsterone, and although the reaction to the *iso*-hormone was slightly less than to 3·5 mg. of androsterone in oil (prostates 37 mg., seminal vesicles 6 mg.), it is doubtful if it was significantly different, especially in view of the larger volume of oil used for the *iso*-hormone. It is hoped to make further experiments on this substance.

VII. EXPERIMENTS ON MICE.

(*a*) *Stimulation of growth in rudimentary or vestigial seminal vesicles*. Attempts have been made to grow the rudimentary (2 mg.) seminal vesicles in mice, some time after prepubertal castration. Crude testis and urine extracts, administered in various forms, have been employed, the mice being usually injected for 10 days before killing. Many of the extracts either were not absorbed or had a deleterious effect on the animals, and insufficient material has been available up to date in a suitable form for the preparation of a dose-response curve on mice. Although it is easy to produce cytological changes in mouse seminal vesicles, such as have been described by Voss and Loewe [1931], the organs have in most experiments shown very small weight increases (up to 8–10 mg.) after 10 days' injection. Such a period is long enough under optimum conditions of dosage to produce seminal vesicles up to 140 mg. Ten days' stimulation of the testes of 10 normal immature 10–15 g. mice by prolan produced seminal vesicles having an average weight of 101 mg. The largest seminal vesicles so far developed in an immature castrated mouse weighed 46 mg.; this animal had received about 100 capon units of a urine concentrate in 10 days. A crude testicular extract produced growth of seminal vesicles to a weight of 35 mg. in another mouse, which was given 6 injections on alternate days and then killed 10 days after the last injection. A group of five immature castrated mice each received a total of 3·5 mg. androsterone in dilute alcohol during 10 days; the seminal vesicles were very small, averaging just over 5 mg., but the epithelium was glandular in comparison with that of a castrated control.

Experiments with crude extracts were also carried out on mice castrated postpubertally at various body weights; in these the seminal vesicles had atrophied completely and weighed 5–20 mg. Such seminal vesicles naturally reached a larger size than those of the immature castrates, but the extent of their reaction was more difficult to gauge owing to the greater variation of the vestigial gland. In these tests seminal vesicles of 30–40 mg. were not uncommonly produced, but the largest ones obtained were only 58 mg. after 10 days' injection with testis extract, and 50 mg. after a total of 16 capon units of "Enarmon", compared with 20 mg. controls. On the whole the larger initial size of the atrophied adult, as against the rudimentary, seminal vesicles, probably does not counterbalance the disadvantage of their greater variability, although the weighing of the seminal vesicles after small doses of male hormone can be more readily carried out.

(*b*) *Maintenance of seminal vesicles in adults.* Several attempts were made to maintain the size of the seminal vesicles for 10 or 14 days in castrated adult mice by means of injections of male hormone and of oestrin begun on the day of castration..

It was assumed from earlier work [Deanesly and Parkes, 1933] that there would be less variation in initial seminal vesicle weight if large mice with a body weight of 24–30 g. were selected for these experiments; they therefore differ from the maintenance experiments on rats (Section V), since they are concerned not with the period of rapid growth, but with the later phase of seminal vesicle enlargement in which the rate of increase is slackening. Unfortunately it is found that in such mice, 10 days after castration, the range of variation in seminal vesicle weight is so great that even in groups of 20, one or two animals in which the seminal vesicles have remained large will materially affect the group average, and in groups of 10 or less only a large difference in seminal vesicle weights from the controls can be considered significant. The results of these experiments, which can only be regarded as preliminary, are set out in Table VI; the controls include those mentioned in an earlier paper

Table VI. *Maintenance of mouse seminal vesicles after postpubertal castration.*

Average normal seminal vesicle weight 246 mg. at an average body weight of 27 g.

No. of exp.	Average body weight at castration g.	Days after castration	No. of mice	Daily injection	Average seminal vesicle weight mg.
Controls	27·5	10	39	None	100
Exp. 20	27·5	10	9	10γ oestriol in aqueous alcohol	72
Exp. 20	27·5	10	10	1γ oestriol in aqueous alcohol	58
Exp. 13	27·0	10	18	1 capon unit ("Proviron") in oil emulsion	114
Exp. 12	25·5	10	20	2 capon units ("Proviron") in oil emulsion	115
Exp. 14	25·5	10	20	5 capon units ("Proviron") in 0·4 ml. oil	124
Controls	25·5	14	25	None	64
Exp. 50	26·0	14	10	1 mg. androsterone in 0·1 ml. arachis oil	117
Exp. 58	30·0	14	3	2 mg. androsterone in 0·2 ml. arachis oil	157

[Deanesly and Parkes, 1933] with some additional mice. In all the experimental groups, except the 3 mice in Exp. 58, the average seminal vesicle weight is nearer to that of the corresponding castrated controls than to that of normal mice of the same body weight. Exp. 20, in which the seminal vesicle weights are below those of the controls, should probably be regarded as negative, since it is unlikely, though not impossible, that injections of oestriol would accelerate the shrinkage of the glands which at this stage is brought about mainly by the loss of secretion [*cf.* Moore and Price, 1932]. Exps. 12–14, 50 and 58, though far from conclusive, seem to show partial maintenance of the glands, and this is corroborated for the last two experiments by the histological appearance of the cranial (anterior) prostate attached to the seminal vesicles. It should certainly be possible to maintain the size of the mouse seminal vesicles after castration by injections of male hormone; the relatively large dose which seems to be needed is probably due to the conditions of absorption from oily solutions which are more unfavourable in the mouse than in the rat.

VIII. EXPERIMENTS ON GUINEA-PIGS.

Two groups of prepubertally castrated guinea-pigs were injected with androsterone alone and in conjunction with oestrone and were killed 24 hours after the last injection; the results are summarised in Table VII. The accessory glands of guinea-pigs, in our experience, are slower to shrink in weight after castration than those of the rat or mouse. After prepubertal castration there seems to be little or no shrinkage of the comparatively undeveloped glands. The high average weight for the prostate in the control castrated animals in Table VII is due to one animal in which it was exceptionally large. On such small numbers the figures for the average weights can only be regarded as approximate, but the results are very consistent. In the two experimental groups the average weights of prostate and seminal vesicles were almost exactly the same; the addition of oestrone had not reinforced the androsterone, and the experiment confirmed the conclusion reached as the result of experiments with rats (Section VI *f*). A total dose of 15 mg. androsterone caused an increase in the seminal vesicles from 40–50 mg. to 120 mg., which suggests that they are more responsive to this substance than are the rat seminal vesicles: the effect however may be due less to greater sensitivity than to the larger size of the rudiment. The prostate had also tripled in size in the experimental guinea-pigs; its average weight, 183 mg., may be compared with the average weight of the prostate, 129 mg., in rats receiving

Table VII. *Average weights of prostate and seminal vesicles in control and injected guinea-pigs.*

No. of animals	Body weight at castration g.	Body weight at death g.	Condition at autopsy	Average prostate weight mg.	Average seminal vesicles weight mg.
4	—	200	Normal immature	57	42
3	200	250–450	21–89 days after castration, not injected	76	53
3	193	473	88 days after castration: had 15 daily injections of 1 mg. androsterone in arachis oil	186	121
3	197	442	88–89 days after castration: had 15 daily injections of 1 mg. androsterone + 10γ oestrone in arachis oil	180	120

the same doses of androsterone over a slightly shorter period (Tables III and V). Allowing for the difference in size of the rudiment, the actual gain in weight in the two species is very similar.

IX. HISTOLOGICAL OBSERVATIONS.

(*a*) *Rats and mice.* The prostate and seminal vesicles of one or more animals from most of the experimental groups were examined microscopically, all material having been fixed in Bouin's fluid as a routine. Sections were cut at 7μ and 10μ and stained in haematoxylin and eosin. Since the histological changes in the rudimentary accessory glands of rats and mice giving a positive response to male hormone have been described in detail in a number of papers by earlier workers already cited, no account of these will be given here. The present observations were intended to be mainly quantitative and histological examination was used as a means of distinguishing any abnormal growth stage that might be produced experimentally, *e.g.* by oestrin injections [David *et al.*, 1934]. The glands from injected rats were therefore compared with normal seminal vesicles and prostates of similar weight, as well as with those of the appropriate castrate controls. Owing to the variations both in the normal growth rate of the accessory organs and in the structure of different parts of the glands, a histological basis for standardising extracts seems undesirable if not impossible. Hansen [1933] has drawn attention to some of the difficulties involved.

Since the rat prostate lobes differ histologically [Walker, 1910], complete serial sections were cut through the glands of a young animal (one in five being mounted). These showed that, at the early spermatogenetic stage examined, three types of glandular tissue could be distinguished in the cranial, dorso-lateral and ventral lobes respectively. Moore, Price and Gallagher [1930] describe the last two as histologically similar but actually, though both are prostatic in type, they can be readily distinguished in section, since there is less fibrous tissue in the ventral lobes and the alveoli are much larger. For routine examination transverse sections were cut through a ventral prostate lobe, the cranial lobe being similarly examined in some rats and in the adult experimental mice.

The general conclusions drawn from the histological observations may be summarised as follows:

(1) In the rat maintenance experiments (Section V) all prostate sections examined appeared to be histologically normal; they showed none of the castration changes described by Moore, Price and Gallagher [1930]. No attempt was made to section the seminal vesicles in these experiments, since the weights seemed to be an adequate guide to their condition.

(2) The developing prostates of the prepubertally castrated rats injected with androsterone were not obviously different in any way from normal glands of the same size. In other words, androsterone, *iso*androsterone and the urine concentrates tested will all cause normal development to a greater or lesser extent in a gland ordinarily controlled by the secretion of the gonad. When oestrin is injected in conjunction with the male hormone, the greater activity of the latter on the prostate tends to mask any oestrin effect. Further, if oestrin is injected alone for 10 days the reaction of the rat prostate is very slight [David *et al.*, 1934] and not easily distinguishable in section.

The largest seminal vesicles grown with androsterone in prepubertally castrated rats weighed only 82 mg.; they appeared to be histologically normal for their size and contained secretion. The largest glands produced by the same dose of androsterone (20 mg.) *plus* 100γ of oestrone weighed 94 mg. and were slightly better developed, but similarly normal. The only essential effect of the oestrone seems to be the acceleration of the earliest stages of seminal vesicle growth by the development of fibrous tissue [David *et al.*, 1934; Korenchevsky *et al.*, 1934, 1, 2]. This is well shown in the rats receiving oestrone alone, where the seminal vesicle epithelium has not reacted. No fresh observations worth recording were made on the seminal vesicles of immature castrated mice receiving male hormone injections. The cranial (anterior), prostate lobes of the mice injected with 1 or 2 mg. of androsterone daily in the maintenance experiments show some alveolar shrinkage as compared with the prostates of normal mice, but the epithelium is comparatively normal and is still secreting.

The adrenals were examined from 7 injected mice and 5 controls from these experiments (Nos. 50 and 58, Table VI) in order to see whether male hormone had inhibited the changes towards a female type of cortex normally following castration [Howard-Miller 1927; Deanesly, 1928]. The zona reticularis, which tends to atrophy in the 21-day male mouse and can hardly be distinguished in the adrenal of the adult, reappears after castration and forms a dark-staining band round the medulla, consisting of small cells free from fatty inclusions. No distinction could be made however between the experimental and control groups. It is probable that the adrenal is not affected directly by the testicular hormone, but only indirectly through the pituitary, and the present injections of androsterone were inadequate to influence the latter gland.

(*b*) *Guinea-pigs.* The seminal vesicles and prostate were examined histologically in one animal from each of the groups listed in Table VII. In the immature 200 g. guinea-pig the seminal vesicles and prostate, though small, are already actively secreting. In the corresponding castrated animal, which has the largest accessory glands in the group, the glandular epithelium appears definitely inert, but its regression is much less conspicuous 89 days after castration than in a similar mouse or rat. The prostatic alveoli are as large as in the corresponding normal animal, but the epithelium is disorganised. In guinea-pigs from both experimental groups, the seminal vesicles and prostate, including the coagulating glands, showed well-marked growth and secretion, and there were no obvious differences between the two. The histological changes were of the usual type, including distention of the prostatic alveoli and of the seminal vesicles, and need not be described in detail.

X. Discussion.

The most striking observation in the course of this work is that, although maintenance of the accessory glands of postpubertally castrated rats was achieved, the highest doses of androsterone administered for 10 days failed to produce much growth of the accessory organs of prepubertally castrated rats. In particular, androsterone seems least effective on the seminal vesicles. Although the growth of these glands normally starts later than that of the prostate, yet in the experimental animals receiving androsterone, the seminal vesicles tended to remain disproportionately small. Moreover, when, as in the 28-day experiment, there was considerable growth of the prostate, that of the seminal vesicles lagged far behind. Even when allowance is made for inefficiency in the form of administration and unsatisfactory absorption, there would be some difficulty in assuming that sufficient androsterone is normally secreted by the testis to account for its male endocrine activity.

A clue to the existence of other active substances is provided by comparison of a concentrate from natural sources with androsterone on the basis of two different responses, namely, growth of the comb in the capon and of accessory organs in the rat. It is then found that one concentrate from urine, "Enarmon", when administered to rats in the same dosage as androsterone, on the basis of capon units, is several times as active as the latter, the contrast being particularly marked in the effects on the seminal vesicles (Figs. 8 and 9). The discrepancy is analogous, in fact, to that which has been observed in the last four years in the comparison of male hormone concentrates from testis and from urine. In 1931, Laqueur, de Fremery *et al.* discussed fluctuations in the ratio rat unit/bird unit in preparations from testis, and, in a later paper Laqueur, Dingemanse *et al.* [1931] speak of a "seminal vesicle hormone". They followed this work by putting forward the view that testicular extracts were superior to urine extracts on mammals [Freud and Laqueur, 1934; Freud, 1934; Dingemanse *et al.*, 1935][1]. The assumption that testicular extracts contain an active substance chemically different from that in urine extracts is confirmed by the work of Gallagher and Koch [1934, 1, 2; *cf.* also Matsuzaki, 1933, 1934; Ogata and Hirano, 1933].

There is some evidence however that crude urine concentrates may also contain a substance active on accessory glands which is lost on further purification. Our own results have shown a significant difference between "Enarmon" on the one hand and "Proviron" and "Hombreol" on the other. Korenchevsky also seems to have detected significant differences between urine concentrates of the same capon titre [Korenchevsky *et al.*, 1933, 1, 2; 1934, 2], though he emphasises rather the superiority of his own method of assay as a measure of male hormone activity than the implication that the materials differ in any essential way. Freud [1933] refers to "the loss of potency with respect to seminal vesicles without corresponding loss of potency in capons" when male hormone extracts are purified. This observation actually led the Amsterdam school to the abandonment, for a time, of the "seminal vesicle hormone" hypothesis, and to its replacement by the hypothesis that synergism of oestrin and male hormone stimulated complete seminal vesicle growth.

Undoubtedly, not only is oestrogenic material produced by the male organism, but oestrin itself has a definite stimulating effect on the fibromuscular tissue of the seminal vesicles [Freud, 1933]. The experiments recorded here however convince us that administration of a mixture of androsterone and oestrone does not reproduce the results of administration of a crude urine concentrate of slight oestrogenic potency. This point may be made clear by reference to Figs. 8 and 9, from which it is seen that with one exception the combination of oestrone with androsterone has little or no extra effect on the accessory organs as compared with androsterone alone. The exceptional case is that of the highest dose of androsterone *plus* oestrone, which caused slightly greater growth of the seminal vesicles (Tables III and V), but this is little more than that provoked by one-fourth the number of capon units administered in the form of "Enarmon". The reaction of the prostate to androsterone over 10 days was not increased by oestrone, and the greater activity of "Enarmon" was still obvious.

One is left therefore with the conclusion that crude urine extracts, and presumably testicular extracts also, contain some other androkinetic substance, either (*a*) supplementary to, or synergistic with, androsterone or (*b*) acting independently of androsterone, possessing a greater ratio of accessory organ activity to comb growth activity than androsterone and being the chief or only male hormone produced by the testis. The comparative success of the maintenance experiments and the nature of the histological findings suggest strongly that androsterone is quantitatively rather than qualitatively deficient and dispose of the necessity for a supplementary or synergistic substance. The existence in the body of a more active form of male hormone present to some extent in urine, and *a fortiori* in testis, is much more likely.

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[1] *Note added to proof.* In a further short note, seen after completion of this paper, David and Freud (*Acta Brev. Neerl.* 1935, 5, 31) compare the activities of androsterone, "Hombreol" and testis extract on the growth of the accessory glands of immature castrated rats. Administered in equal capon unit dosage, the first two are of equal potency whereas testis extract is much more active, particularly on the seminal vesicles.

An analogy may be drawn between the male and female hormones in this connection. In each case the characteristic hormonal activity can be exercised by more than one compound. Oestrone and oestriol, both capable of producing the same effects, have a different ratio of activity on different test-objects, *e.g.* cornification of the vagina of the immature rat and of the ovariectomised adult. Oestradiol (so-called "dihydro-oestrin") appears to be the most active oestrogenic compound, and it is likely that it is the primary form of the oestrogenic hormone in the body, since it can be extracted from ovaries [Doisy *et al.*, 1935]. The artificially prepared dihydroxy-derivative of androsterone possesses a similarly enhanced activity in capons [Ruzicka *et al.*, 1935] but there is as yet no evidence of its natural occurrence or of its relative activities in mammals and birds. The analogies between the two hormones suggest strongly the possibility that there remains to be isolated from natural sources some derivative of androsterone which possesses not only a higher degree of activity but also a different ratio of activity on accessory organs in the mammal and on the comb of birds.

SUMMARY.

1. Androsterone, *iso*androsterone, and various urine concentrates have been tested on the accessory reproductive glands of castrated rats, mice and guinea-pigs.

2. Androsterone was injected into groups of adult rats for 14 days, beginning on the day of castration; 1 mg. daily was enough to maintain growth and normal structure in the prostate of 140–160 g. rats but more than 2 mg. daily were required for maintenance of the seminal vesicles. Larger rats, castrated and injected daily with 3 mg. androsterone, had prostates above the normal size and normal seminal vesicles.

In similar experiments with mice 1 and 2 mg. daily of androsterone failed to maintain completely the seminal vesicles or the cranial lobes of the prostate.

3. Androsterone, in both oily and alcoholic solution, was injected into groups of immature castrated rats for 10-day periods in an attempt to grow the rudimentary accessory glands; on a total dose of 20 mg. of androsterone the largest prostate produced in these experiments was 212 mg. or about a quarter of the full size, and the largest seminal vesicles were 82 mg., about one-tenth of the full size. In most of the androsterone experiments however, particularly with oily solutions, the seminal vesicles of the rats gave a very poor reaction as compared with the prostate. This was particularly obvious in a 28-day experiment; on 1 mg. daily the prostate reached a maximum of 433 mg. whilst the largest seminal vesicles were only 63 mg.

4. A comparison of different urine concentrates on the basis of the number of capon units contained in each showed that, whereas in two of them the effect per capon unit on the accessory glands of rats was much the same as that produced per capon unit (100γ) of crystalline androsterone, in the third urine preparation the activity on rats was more than three to four times as great.

5. It was shown that the activity of the most effective urine concentrate could not be reproduced by combined administration of oestrone and androsterone, which gave results little, if any, better than androsterone alone.

6. Experiments were also carried out with castrated mice and guinea-pigs.

7. It is concluded that certain urine concentrates may contain a male hormone other than androsterone, with a much greater activity per capon unit on the accessory glands of mammals.

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REFERENCES.

David, Freud and de Jongh (1934). *Biochem. J.* **28**, 1360.
Deanesly (1928). *Proc. Roy. Soc. Lond.* B, **103**, 523.
—— and Parkes (1933). *J. Physiol.* **78**, 442.
—— and Rowlands (in preparation).
Dingemanse, Freud and Laqueur (1935). *Nature*, **135**, 184.
Doisy, MacCorquodale and Thayer (1935). *Weekly Bull. St Louis Med. Soc.* **29**, March 22nd.
Engle (1926). *Anat. Rec.* **34**, 75.
Freud (1933). *Biochem. J.* **27**, 1438.
—— (1934). *Acta Brev. Neerl.* **4**, 145.
—— de Fremery and Laqueur (1932). *Pflüger's Arch.* **229**, 763.
—— de Jongh, Laqueur and Münch (1930). *Klin. Woch.* **9**, 772.
—— and Laqueur (1934). *Acta Brev. Neerl.* **4**, 100.
Gallagher and Koch (1934, 1). *Endocrinology*, **18**, 107.
—— —— (1934, 2). *J. Biol. Chem.* **104**, 611.
Girard and Sandulesco (1934). Brit. Patent Appl. No. 6640, 1st March.
Greenwood, Blyth and Callow (1935). *Biochem. J.* **29**, 1400.
Hansen (1933). *Endocrinology*, **17**, 163.
Howard-Miller (1927). *Amer. J. Anat.* **40**, 251.
Koch (1932). Sex and internal secretions. Ed. E. Allen, Ch. 8. (Baltimore.)
Korenchevsky (1935). *Nature*, **135**, 434.
—— and Dennison (1934, 1). *Biochem. J.* **28**, 1474.
—— —— (1934, 2). *Biochem. J.* **28**, 1486.
—— —— and Kohn-Speyer (1932). *Biochem. J.* **26**, 2097.
—— —— —— (1933, 1). *Biochem. J.* **27**, 557.
—— —— —— (1933, 2). *Biochem. J.* **27**, 778.
Laqueur, Dingemanse, de Fremery, Freud, de Jongh, Kober, Luchs and Munch (1931). *Nederl. Tijdschr. Geneesk.* **1**, 1648.
—— de Fremery, Freud, de Jongh, Kober, Luchs and Münch (1931). *Ber. ges. Physiol.* **61**, 364.
Martins (1930). *Endokrinologie*, **7**, 180.
Matsuzaki (1933). *Proc. Imp. Acad. Tokyo*, **9**, 342.
—— (1934). *Jap. J. Med. Sci.* iv, Pharm. **8**, 31.
Moore and Gallagher (1930, 1). *Amer. J. Anat.* **45**, 39.
—— —— (1930, 2). *J. Pharm. Exp. Ther.* **40**, 341.
—— Hughes and Gallagher (1930). *Amer. J. Anat.* **45**, 109.
—— and Price (1932). *Amer. J. Anat.* **50**, 13.
—— —— and Gallagher (1930). *Amer. J. Anat.* **45**, 71.
Ogata and Hirano (1933). *Proc. Imp. Acad. Tokyo*, **9**, 345.
Ruzicka (1935). *Naturwiss.* **23**, 44.
—— Goldberg and Brüngger (1934, 1). *Helv. Chim. Acta*, **17**, 1389.
—— —— and Meyer (1935). *Helv. Chim. Acta*, **18**, 210.
—— —— —— Brüngger and Eichenberger (1934, 2). *Helv. Chim. Acta*, **17**, 1395.
Schoeller and Gehrke (1931). *Wien Arch. inn. Med.* **21**, 329; *cf. Zahnärztl. Mitt.* No. **13**, March 29th.
Vavon (1914). *Ann. Chim.* (ix), **1**, 146.
Voss and Loewe (1931). *Arch. exp. Path. Pharm.* **159**, 532.
Walker (1910). *Johns Hopkins Hosp. Bull.* **21**, 182.

CLXXV. DIFFUSION EQUILIBRIA FOR THE ISOLATED FROG'S KIDNEY.

II. UREA.

By EDWARD JOSEPH CONWAY AND FRANK KANE.

From the Department of Physiology and Biochemistry, University College, Dublin.

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In a previous communication [Conway and Kane, 1934] it was shown that urea existed in the normal frog's kidney considerably in excess of that in the blood or of the sartorius muscle. This latter was found to have a urea concentration which might be expected to arise from a passive equilibration with the blood, the urea diffusing into all the water of the tissue.

It was also shown that the urea in the normal frog's kidney diffused outwards at the same rate as it diffuses into or out of muscle or into the inactive kidney. The diffusion coefficient of urea was found to be the same for the kidney as for muscle within the error limits of such determinations, its value being $1 \cdot 1 \times 10^{-5}$ (cm.^{2}/min.).

In the present investigation the point of chief interest was to discover the urea diffusion equilibria for active and inactive kidneys immersed in urea solutions. It will be shown that urea passes from urea-Locke solutions into active kidneys having a lower urea concentration until the total concentration of the tissue far exceeds that of the external fluid. When the tissue is rendered inactive by cyanide the urea enters only to the extent that may be expected for a passive equilibration with the tissue water.

In this process a freely soluble substance is being concentrated by the active kidney and the form in which it is concentrated appears to leave it in free solution and in such a condition that it can diffuse outwards at the same rate as urea from the inactive kidney or from muscle.

The facts of this urea diffusion are of such importance for renal theory that the method used for the analyses should be very carefully certified to give accurate data for the true urea content. There is all the more reason for this as the urease ferment extracts as ordinarily prepared may react to produce ammonia with the frog's kidney in addition to the urea ammonia and the free ammonia which is formed in all such surviving tissues.

It will be shown that bringing the kidney suspension to the boiling point in a small tube, and then immersing this in boiling water for five minutes completely eliminates the production of "extra" ammonia (*i.e.* other than urea ammonia) by the urease ferment. The procedure also stops any further production of free ammonia in the tissue suspension itself.

The results so obtained with the urease method used on boiled tissue suspension have been compared with xanthydrol determinations in a sufficient number of analyses to show that both methods give identical results.

The nature of the "extra" ammonia formed by urease ferment extracts on fresh unboiled renal suspension is in itself of much interest, and though not concerning us particularly in this communication, some of the main conditions of its formation will be necessarily demonstrated in a critical analysis of the urease method.

As a result of this investigation of method it might be inferred that the results given in the previous communication were a little too high. The error would not exceed 7 mg. per 100 mg. kidney, and this amount does not invalidate any of the conclusions there drawn. It necessitated a further investigation of the diffusion coefficient. We expected to find this somewhat higher than in the last determination—namely about $1{\cdot}2\times10^{-5}$, instead of $1{\cdot}1\times10^{-5}$. However, the repeated determinations in November and December gave again the same figure (in fact very slightly less). We may explain this by supposing that the "extra" ammonia in the earlier experiments was, as previously considered, entirely negligible.

METHODS.

(a) *Urease method.*

The method used has been described in detail in previous communications [Conway, 1933; Conway and Kane, 1934]. In the present determinations a weighed amount of tissue was ground with a measured volume of water and a little pure quartz sand (Merck) in an agate mortar. The quantities used were such that 0·5 ml. of the centrifuged ground mixture contained as a rule about 5–20 mg. tissue. The fluid was decanted after centrifuging to another rather narrow tube, and after the level had been marked was brought momentarily to the boiling point. The tube was then immersed in a beaker of boiling water for five minutes, being covered with a watch glass. Any fluid loss was made up after cooling—the loss being generally negligible.

0·5 ml., as an aliquot part of the fluid, was transferred to the outer chamber of an absorbing "unit", the inner chamber containing 1 ml. 0·001 *N* sulphuric acid (made up with 20 % alcohol and containing methylene blue and methyl red [Conway and Byrne, 1933]. The remaining procedure has been already described in detail. The titrations at the end were carried out with 0·005 *N* carbon dioxide-free alkali from the horizontal micro-burette previously described [Conway, 1934].

The accuracy of the method. It has been already shown [Conway, 1933] that the degree of hydrolysis of the urea by the urease ferment, and the absorption of the formed ammonia proceed to 99 % at least of the theoretical. The relative absorption rate for ammonia quantities of say 10 γ ammonia-nitrogen is the same as for 10,000γ. The variable error of ammonia determinations down to quantities of 10γ ammonia-nitrogen was approximately 0·5 % expressed as a coefficient of variation. The determination of urea quantities of the order of 40γ urea-nitrogen had a coefficient of variation of 1·2 %. The slight difference may be attributed in part to the use of 1 ml. of fluid in one procedure and only 0·2 ml. in the latter. No very appreciable variable error is therefore introduced by the hydrolysis of the urea itself.

In the present investigation we are concerned with the analyses of urea or ammonia quantities of the order of 3γ of nitrogen or less. From an examination of the analyses of the ammonia content of the stock urease extract an excellent opportunity presents itself of examining the variable error in the routine determination of urea- or ammonia-nitrogen of about 1γ. In four experiments following on one another 20 such determinations were carried out. The average nitrogen analysed was 0·65γ. Eighteen of the results did not deviate from this figure by more than 0·1γ, the standard deviation of the series being 0·06γ. It is possible to reduce the error further by slower titrations and stricter attention to the practically complete absence of carbon dioxide from the titrating alkali. Without this refinement however the accuracy attained is quite sufficient for the present investigation and represents no more than 3–5 % error in the analysis of 3 γ of ammonia- or urea-nitrogen.

We doubt if any other method could compare with this for combined accuracy, simplicity and ease of working with such minute quantities of ammonia. The present and the previous communication are based on over 1000 such analyses.

The question of the "extra" ammonia produced by the urease extract. The various ammonia fractions (other than the reagent ammonia) that arise in a urease analysis of fresh kidney suspension may be listed as follows:

(1) Ammonia from the urea in the frog's kidney.

(2) Ammonia from such precursors as adenylic acid and formed in all such surviving tissues, the amount increasing with the time after removal.

(3) Ammonia formed from an interaction between some substance in jack bean extract interacting with some substance other than urea in the kidney.

To investigate the amounts and changes of these quantities during the course of extended incubation the following experiments were carried out, the volume and reaction of the analysed mixture being in each case the same. Groups of units were set up for the addition of 1 ml. of saturated potassium carbonate after various incubation times (conducted at 37°). These units contained the following mixtures in the outer chamber:

I. 0·5 ml. neutral phosphate (0·05 *M*).
0·5 ml. of centrifuged kidney suspension (containing about 5–20 mg. per ml.).
0·5 ml. glycerol (diluted 1 in 20).
0·5 ml. water.

I *a*. Same as I except that the centrifuged suspension was boiled in the manner already described.

II. 0·5 ml. of glycerol-urease extract (diluted 1 in 20).
0·5 ml. centrifuged kidney suspension.
0·5 ml. neutral phosphate (0·05 *M*).
0·5 ml. water.

II *a*. Same as II except that boiled centrifuged kidney suspension was used.

Similar units were set up containing the phosphate, glycerol and urease in the same volume of fluid, and also containing merely the phosphate and glycerol in order to ascertain the reagent ammonia.

Subtracting the reagent ammonia in each case, the ammonia from I and I *a* gives the free ammonia in the unboiled kidney suspension and the boiled respectively. Subtracting these values as well as the reagent ammonia from II and II *a* respectively one obtains the ammonia produced by the interaction of the urease ferment extract with the unboiled and boiled kidney suspensions.

Fig. 1 shows the mean results obtained in five experimental series, in each of which the incubation periods (before the addition of saturated carbonate) were maintained from 15 minutes to 3 hours.

A number of conclusions may be drawn from these results.

(1) After 15 minutes' incubation with urease all further ammonia formation stops in the boiled kidney suspension. The ammonia so formed (the original free ammonia being allowed for in Fig. 1) represents beyond any doubt the true urea content of the tissue and is identical with that found in xanthydrol determinations.

(2) The ammonia formed by the action of the urease extract on the fresh kidney suspension shows a linear increase up to 90 minutes' incubation and then remains steady up to 180 minutes. Extrapolation to zero time meets the line for the boiled suspension.

(3) The free ammonia of the boiled suspension also does not alter with incubation.

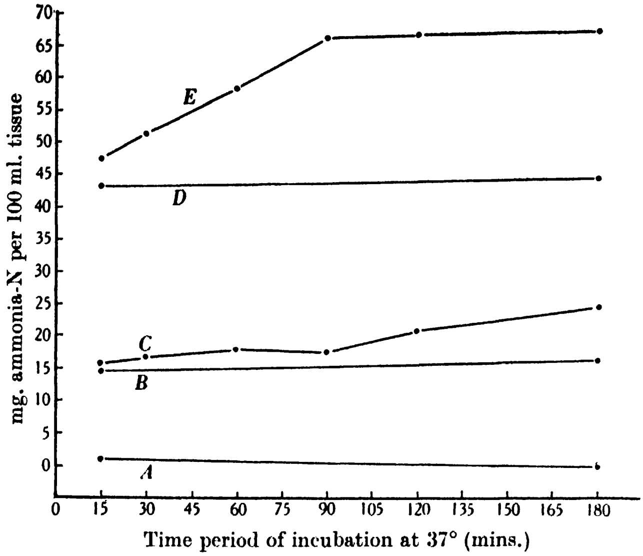

Fig. 1. The experiments from which the above figure is drawn are described in the text. The points are the means obtained in five experimental sets. *A*, Ammonia-N formed by interaction of boiled urease and kidney suspension; *B*, free ammonia-N in boiled suspension; *C*, free ammonia-N in unboiled suspension; *D*, ammonia-N formed by interaction of urease and boiled kidney suspension; *E*, ammonia-N formed by interaction of urease and unboiled kidney suspension.

(4) The free ammonia of the unboiled suspension rises slightly as the incubation time increases to 90 minutes and then begins to rise steeply. Clearly some marked change occurs around this period, with unboiled suspension under the above conditions. (The further precise elucidation of the "extra" ammonia formation by the interaction of the fresh kidney suspension and the urease extract does not concern us here. As a working hypothesis we may suppose however that a ferment such as arginase or a deaminase exists also in the urease extract and acts on arginine or a similar substance produced by proteolysis in the incubated tissue. Some further facts may be mentioned in support of this hypothesis. Doubling the concentration of the urease doubles the rate of formation of this "extra" ammonia. Doubling the concentration of the renal suspension causes the same result. Boiling the urease extract eliminates any ammonia formation due to the interaction of the extract and the suspension.)

(*b*) *Xanthydrol method.*

The xanthydrol method was also used in a certain number of analyses of kidney suspensions, diffusates and blood from the same frogs, to confirm further the findings of the urease method. In using this method 4 ml. of the renal suspension or diffusate containing approximately 1 mg. urea per 100 ml. were measured into a 15 ml. centrifuge-tube, and 1 ml. of the Tanret-Polonowski reagent was added (tungstic acid precipitation was used for the first experiment). The tube was centrifuged for 15 minutes, 4 ml. of the clear fluid being taken in another 15 ml. centrifuge-tube and 4 ml. of a xanthydrol mixture (freshly formed by the addition of 5 parts of 5 % xanthydrol in methyl alcohol to 3 parts of glacial acetic acid) added, the fluids being mixed with a thin glass rod. The mixture was allowed to stand overnight. It was then centrifuged for 15 minutes. The fluid was very carefully decanted and the tube allowed to drain upright for a few minutes on absorbing filter-paper. 2 ml. methyl alcohol saturated with dixanthydryl urea were then added to the tube and the solid residue was stirred up. The tube was again centrifuged and the procedure repeated. After the final centrifuging the draining was allowed to continue for 5 minutes and then the end of the tube wiped with filter-paper. Up to this point the method used was largely similar to that used by Rehberg *et al.* [1932]. The further procedure consisted in adding 2–5 ml. of 50 % sulphuric acid and stirring with a glass rod. This is an adaptation of the method used by Beattie [1928].

After some time the yellow colour produced was examined in a micro-colorimeter, using a standard formed by dealing in an exactly similar manner with 4 ml. of a 1 mg. per 100 ml. urea solution instead of the kidney suspension. Suitable blanks were also carried out. Duplicate analyses were made in each case and colorimeter readings taken by independent observers.

The normal relation between the urea concentrations in the blood and kidney of the frog (Rana temporaria).

The relation found in the spring frogs has a mean value of 45 mg. per 100 ml. blood to 85 mg. per 100 ml. in the kidney. A new series of 14 analyses, with the revised technique, on boiled kidney suspensions gave an average of 82 mg. per 100 ml. The corresponding blood values (for 10 experiments) were higher for these months (November to February) than in summer, giving an average value of 56 mg. urea per 100 ml. The individual results are given in Table I. For the blood analyses 0·1 ml. was taken from each frog and mixed, the analysis being subsequently carried out on the mixed blood.

In a series of seven experiments not only was the urea in the boiled kidney suspensions and corresponding blood samples determined, but also the amount that diffused freely outwards in two hours from the companion control kidneys. These were immersed in about 50 times their volume of Locke solution and stirred by a stream of oxygen. The average value for urea in the kidneys of this series was found to be 85 mg. per 100 ml. The analyses of the diffusate showed that on the average 75 mg. urea per 100 ml. kidney diffused out in two hours into the Locke solution. One set (No. 5 in Table I) shows an aberrant figure for the renal urea in relation to the diffused quantities. Omitting this as probably erroneous the average values are 83 mg. urea per 100 ml. for the kidney and 76 mg. urea diffused per 100 ml. The average blood value in these six sets was 55 mg. per 100 ml. An excess of 40 % over the blood urea value has therefore diffused freely from the kidneys in two hours.

An average value of 1·3 mg. urea per 100 ml. was present in the Locke solution at the end of the diffusion period and this we may presume to be in equilibrium with about 2 mg. per 100 ml. of kidney tissue. The total diffusing concentration was therefore 81 mg. urea per 100 ml., so that 94 % of the total possible has diffused in two hours (practically the same figure is maintained if we include two extra sets for which the blood analyses are not available, the average percentage diffused is then in fact 95.

Table I.

No. of exp.	No. of frogs	Av. wt. of single kidney mg.	Kidney (boiled suspension) mg. urea per 100 ml.	mg. urea diffused per 100 ml. in 2 hrs. Calctd. from diffusate	mg. urea diffused per 100 ml. calc. from boiled diffusate (2 hrs.)	Kidney after diffusion (boiled suspension) mg. urea per 100 ml.	Blood mg. urea per 100 ml.	Blood (boiled) mg. urea per 100 ml.	Kidney diffusate mg. urea per 100 ml.
1	3	40	66	59	58	8	30	—	1·1
2	3	35	125	107	—	5	86	—	1·9
3	3	43	77	72	75	—	62	—	1·4
4	3	38	85	87	92	—	68	68	1·6
5	3	36	105	70 (?)	73	—	54	54	1·2
6	4	25	82	75	81	—	—	—	1·2
7	2	53	70	59	59	—	—	—	—
8	5	40	70	61	—	—	36	—	1·2
9	4	41	65	64	—	—	48	—	1·0
10	3	30	79	—	—	—	—	—	—
11	4	25	82	—	—	—	—	—	—
12	3	22	66	—	—	—	—	—	—
13	2	65	93	—	—	—	—	—	—
14	2	71	79	—	—	—	—	—	—

The amount of tissue analysed ranged from 7 to 13 mg. in 0·5 ml. of suspension. Where diffusions (2 hours' duration in Locke, stirred with oxygen) were carried out, one kidney from each frog was taken and the group transferred to Locke, the companion tissues being ground to a suspension in the usual way and analysed. For further details see text.

The average weight of the kidneys examined was 40 mg. and the theoretical amount that would diffuse in two hours—the diffusion coefficient being $1{\cdot}1 \times 10^{-5}$ is 98 %. The difference between the actual and the theoretical is therefore insignificant, and, as shown in section III, after three hours' diffusion 97 % is recoverable from the Locke fluid.

Table II.

No. of frogs	Av. wt. of single kidney mg.	Urea conc. of kidney mg. per 100 ml.		Urea diffused in 2 hrs. mg. per 100 ml. of kidney		Urea conc. of blood mg. per 100 ml.	
		Urease	Xanthydrol	Urease	Xanthydrol	Urease	Xanthydrol
6	51	—	104	—	101	—	72
5	40	70	69	61	59	36	36
4	41	65	66	64	61	48	49

One kidney from each frog was grouped and a suspension made in 12 ml. water; 4 ml. quantities were taken for the xanthydrol analyses, 0·5 ml. quantities were taken for the urease analyses. For the analysis of the diffused quantities the companion tissues were immersed in 12 ml. of Locke and stirred with a stream of oxygen for 2 hours, the Locke fluid being subsequently analysed. 0·1 ml. blood was taken from each frog and the mixed blood analysed.

The first experiment with the kidneys from 6 frogs was carried out in December, the remaining experiments in February from fresh consignments.

The ratio between the blood urea and the renal urea in the summer frogs was found to be 1 : 1·89, the ratio for the winter frogs 1 : 1·49, and as we have seen, nearly the entire amount of the renal urea freely diffuses outwards. Three sets of experiments in which the xanthydrol method was used with duplicate analyses, gave a ratio of blood to kidney urea of 1: 1·43 for winter frogs. The average urea content of the kidney was 80 mg. per 100 ml. and the average diffused quantity into a large volume of Locke 74 mg. urea per 100 ml. tissue. Using the same considerations as above this means that 95 % of the total possible has diffused, the theoretical quantity being again 98 %. Table II contains the details of these experiments and shows that in two sets where series of corresponding analyses were made with the urease method, practically identical results were obtained.

The xanthydrol results are therefore in complete agreement with the urease figures.

Diffusion relations of urea for the isolated frog's kidney.

(*a*) *The diffusion coefficient.* It was considered advisable to make a repeated determination of this coefficient with the revised technique. In the method adopted 12 kidneys were immersed in 20 ml. cyanide-Locke (cyanide concentration—0·02 % KCN) and kept stirred with a stream of oxygen saturated with water vapour.

0·5 ml. was removed at varying intervals ranging from 5 to 180 minutes. Over this period about three additional quantities of 0·5 ml. were removed to determine the free ammonia content. The whole course of the diffusion could be examined from the one group of kidneys without the need of comparing with the companion tissues for each determination after a given time period. The results are given in Table III. The mean value of the diffusion coefficient up to a period when 70 % was diffused was found to be $1{\cdot}09 \times 10^{-5}$, showing therefore no change from the previous determination and indicating that the analyses summarised in the previous paper, from which the diffusion coefficient was determined, were inappreciably different from the true figure. Table III shows also that the whole course of the diffusion is very close to the theoretical curve based on a coefficient of $1{\cdot}095 \times 10^{-5}$ and that after three hours' diffusion 97 % of the theoretical is recoverable.

Table III.

Diffusion period mins.	Amount diffused per 100 ml. kidney mg.	Amount diffused calculated from diff. coeff. of $1{\cdot}095 \times 10^{-5}$ mg. per 100 ml. kidney	Diffusion coeff. calculated from the diffused quantities (cm.^{2}/mins.)
5	25·2	23·6	$1{\cdot}26 \times 10^{-5}$
10	32·9	33·7	$1{\cdot}03 \times 10^{-5}$
20	45·3	48·0	$0{\cdot}99 \times 10^{-5}$
30	57·8	57·1	$1{\cdot}10 \times 10^{-5}$
60	71·0	73·3	—
180	78·0	81·3	—

Temperature, 17°.

In the above experiment 12 kidneys from six frogs of one consignment and weighing 533 mg. were introduced into 20 ml. Locke's fluid and stirred with oxygen. 0·5 ml. quantities were removed at definite times for the urea analysis, three extra quantities of 0·5 ml. being taken over the three hours' period of the immersion for determination of the ammonia content. The kidneys at the end were also analysed, being ground up in 3 ml. of water.

The urea content of the kidneys at the beginning of the immersion was calculated from the analyses as 84·3 mg. per 100 ml. At the end the Locke fluid had a concentration of 2·25 mg. urea per 100 ml., the diffusing concentration therefore being approximately 82 mg.

The slight difference at the end of the experiment between the actual and theoretical quantities diffused may be attributed to the swelling of the kidneys.

(*b*) *Urea diffusion equilibria for the isolated kidney.* Before immersing the isolated kidneys in urea-Locke solutions they were previously washed in ordinary Locke for 20–30 minutes. In this way the concentration of urea was reduced from approximately 80 mg. to about 30 mg. per 100 ml. One of each pair of kidneys were then grouped together and analysed, the companion tissues being immersed and stirred with oxygen in urea-Locke and urea-cyanide-Locke solution respectively. In this re-immersion—if we consider the diffusion coefficient of $1{\cdot}1 \times 10^{-5}$—it would take over two hours before a practically complete equilibrium would occur; after this time there appeared some danger of the activity of the kidney being much reduced. For this reason the time of re-immersion in the urea-Locke was confined to 30–45 minutes, the movement of the urea being studied over this period. The results of this study are sufficient and conclusive in establishing the main point of interest—namely, the concentration of urea by the active kidney from an external fluid. That the time was insufficient for establishing full equilibrium only lends emphasis to the results. At the same time it may be pointed out that about 70–80 % of the total movement of the urea towards equilibrium will have occurred and that from a consideration of the diffusion coefficient the equilibrium figure may be calculated.

A large number of experiments of this type were collected before the revised technique was set going. The individual results were obtained from the one pair of kidneys, one kidney being analysed after the 30-minute washing period and the other immersed for a further 30 minutes in urea-Locke (or urea-cyanide-Locke), the kidneys being stirred in all cases with a stream of oxygen. The concentration of the urea in the external Locke was either 20, 40 or 80 mg. per 100 ml. The results are summarised in Table IV.

Table IV.

		Inactive kidneys (cyanide)			Active kidneys			
Conc. of urea in the urea-Locke mg per 100 ml.	Av. wt of single kidney mg.	No. of exps.	Mean conc. before immersion in urea-Locke mg. urea per 100 ml.	Mean conc. after immersion in urea-Locke mg urea per 100 ml.	No of exps.	Mean conc before immersion in urea-Locke mg. urea per 100 ml	Mean conc. after immersion in urea-Locke mg. urea per 100 ml.	Increase in mean conc of active over inactive kidneys mg urea per 100 ml
20	40	4	27	24 (21–26)	6	31	41 (27–71)	+17
40	33	6	31	37 (30–48)	14	33	50 (31–103)	+13
80	45	5	29	56 (51–60)	17	26	72 (42–104)	+16

In each experiment one kidney from one frog was analysed after the washing period of 30 minutes in Locke, and the companion kidney after the immersion period in urea-Locke.

The mean value of the urea content of the kidneys after the 30-minute washing is in all the groups within the range 30 ± 4 mg. per 100 ml. After re-immersion for 30 minutes in Locke containing 20, 40 or 80 mg. urea per 100 ml. the mean values for the inactive (cyanide) kidneys were 24, 37 and 56 mg. urea per 100 ml. respectively. The mean values for the active kidneys re-immersed in Locke containing the same urea concentration, but without cyanide, were 46, 50 and 72 mg. per 100 ml. In each group, therefore, a large excess of urea has diffused into the active kidney in comparison with the inactive kidney. It will be seen from the table that after re-immersion in urea-cyanide-Locke containing 20 mg. per 100 ml. urea the value falls over the period from a mean value of 27 mg. urea per 100 ml. to a mean value of 24 mg., whereas the urea content for the active kidneys rises from 31 to 46 mg. urea per 100 ml.

After immersion in the 80 mg. per 100 ml. urea solution, the content of the active kidney increases on the average from 26 to 72 mg. per 100 ml. There is here insufficient time for the value of the kidney to exceed that of the external fluid, but it exceeds the corresponding value for the inactive kidney by 16 mg. urea per 100 ml.

The question arises—does the movement of urea in the inactive kidneys indicate a passage towards a mere passive equilibrium? Considering the average weights of the kidneys examined in the three groups of cyanide-urea immersions, namely, 40, 33 and 45 mg., it may be calculated from the diffusion coefficient ($1{\cdot}1 \times 10^{-5}$) that in 30 minutes 72, 76 and 70 % of the equilibrium amount will have diffused. From these figures and those in Table IV the equilibrium values for the three groups of cyanide immersions will be 23, 39 and 68 mg. per 100 ml. (the values after 30 minutes being 24, 37 and 56). From a mere passive equilibrium we should expect 17, 34 and 67 mg. urea per 100 ml. The experimental values exceed the theoretical therefore by +6, +5 and +1 mg. urea per 100 ml. or an average of +4 mg. per 100 ml.

Apart from the fact that this quantity is in itself small, it is probably due to an analytical error arising from the "extra" ammonia formed after 15 minutes' action of the urease extract on the kidney suspensions.

Applying the same calculations to the three groups of active kidneys, the equilibrium values are 51, 55 and 95 (the 30-minute values being 46, 50 and 72). These values exceed those for a passive equilibrium (17, 34 and 67) by +34, +21 and +28 mg. urea per 100 ml. They are of about the same order as the difference in urea concentration between the normal frog's kidney and that of the blood, namely 40 mg. per 100 ml. found for the summer frogs and 27 per 100 ml. for the winter frogs.

In the above analyses we were concerned with individual tissues the average values of which are summarised. The individual results, particularly of the active kidneys, gave, as may be expected, rather wide variations. A further group of five experiments was carried out with the revised technique, groups of three kidneys being used for each analysis. The conditions in these experiments were made as uniform as possible in comparing the active with the inactive kidneys. In each experiment of this group 6 pairs of kidneys were taken from six frogs of the same consignment and selected for their similarity in size. The 6 pairs were washed in Locke, being stirred with oxygen for 20 minutes. Three of the pairs were divided (all the pairs were held together by a small amount of uncut tissue) and three of the kidneys taken for analyses, the remaining three being immersed in Locke containing 40 mg. urea per 100 ml. The three remaining pairs were treated similarly, the companion tissues being immersed in cyanide-Locke containing also 40 mg. urea per 100 ml. The kidneys were immersed in the respective urea solution for 45 minutes, being subsequently analysed.

Table V.

			Inactive kidneys (cyanide)		Active kidneys		
No. of exp.	Av. wt of single kidney mg.	Temp.	Conc. of urea in 3 kidneys before immersion in urea-Locke mg per 100 ml.	Conc. of urea in companion tissues after immersion in urea-Locke mg. per 100 ml.	Conc. of urea in 3 kidneys before immersion in urea-Locke mg. per 100 ml.	Conc. of urea in companion tissues after immersion in urea-Locke mg. per 100 ml.	Increase in mean conc. of active over inactive kidneys mg. urea per 100 ml.
1	31	12	32	33	32	42	+ 9
2	31	17	27	42	30	51	+ 9
3	44	18	32	35	30	47	+12
4	39	19	37	34	39	57	+23
5	35	19	33	34	40	59	+25

The experiments are described in the text. In the table the results are arranged in order of the room temperatures at which the experiments were conducted.

Each analysis in the above table has been determined from a group of three kidneys.

The results are shown in Table V and completely confirm the previous results. After the 20 minutes' washing the average value of the companion tissues to those immersed subsequently in the cyanide-urea-Locke is 32·2 mg. urea per 100 ml. After immersion the average is 35·6. The average weight of the kidneys is 36 mg. After 45 minutes with a diffusion coefficient of $1{\cdot}1 \times 10^{-5}$, 86 % will have diffused, so that the final equilibrium value should be 36·2—very slightly above the 45-minute figure. The amount for a purely passive equilibrium is

34 mg. urea per 100 ml. This is in fact the figure reached in four out of five of the experiments, the remaining figure of 42 mg. per 100 ml. being somewhat aberrant.

Reviewing now the active kidneys of this series, in each experimental group the value is much higher than for the inactive kidneys, the difference ranging from 9 to 25 mg. urea per 100 ml. with a mean value of 16 mg. It may be noted that the lower figures of the group are associated with low external temperatures.

Prior to immersion in urea-Locke the average value for the companion (control) tissues of the active kidneys is 34 mg. urea per 100 ml., the average after immersion being 51 mg. urea per 100 ml.

Applying the same calculation as above, with the cyanide kidneys the equilibrium value appears as 54 mg. urea per 100 ml. or 18 mg. in excess of the 36 mg. found with the cyanide kidneys.

Discussion.

Here we may emphasise the fact that the concentration of urea by the active isolated frog's kidney from an external fluid has been definitely proved, the formation of a glomerular fluid by filtration during this process of concentration being an impossibility.

The proof depends on a large number of experiments, and selecting one group of these for illustration, it is briefly as follows. The kidneys of a number of frogs were removed and washed in Locke's fluid for 20 minutes, being stirred—as in all the immersions described here—by a stream of oxygen. Their concentration was thereby reduced to 34 mg. urea per 100 ml. Some were now immersed in Locke containing 40 mg. per 100 ml. of urea, and others in a similar urea-Locke containing cyanide, the immersion being continued for 45 minutes. The resulting concentration of the inactive (cyanide) kidneys was 35·6 mg. urea per 100 ml., that of the active kidneys being 51 mg. urea per 100 ml. Urea has, therefore, been concentrated from an external fluid by the active kidneys, but not by the inactive.

To reconcile these facts with the theory of urea concentration by water reabsorption, we believe that only one explanation is possible, and we shall show that this explanation must in turn be definitely abandoned.

It may be held that after the 20-minute washing period in Locke when the concentration of the kidneys is 34 mg. urea per 100 ml. about 16 mg. of this urea are present in the urine contained within the uriniferous tubules, and that during the remaining 45 minutes' immersion in the urea-Locke this is held there, whereas with the immersion in urea-cyanide-Locke it is practically all released, the external urea reaching then a mere passive equilibrium with the urea of the renal cells. This it might be alleged would account for the difference, so that the excess urea in the active kidneys was not concentrated from an external fluid, but was held there from the beginning of the experiment, in the urine of the tubules. This explanation involves, as a necessary consequence, that if, for the 45-minute period of re-immersion, the Locke and cyanide-Locke fluids contained no urea, then the same difference as before must be manifested at the end between the active and inactive tissues, about 16 mg. per 100 ml. should be retained by the active tissue in excess of the inactive tissue. We have, therefore, conducted a series of experiments to decide this point (which might likewise have been refuted from other available facts—but not perhaps so directly or simply).

Table VI.

Period of re-immersion in Locke after preliminary washing mins.	Active kidneys		Inactive kidneys (cyanide)		Increase in mean conc. of active over inactive kidneys mg. urea per 100 ml.
	Av. wt. of single kidney mg.	mg. urea per 100 ml. after re-immersion	Av. wt. of single kidney mg.	mg. urea per 100 ml. after re-immersion	
20	26	21	27	18	+3
20	22	19	23	18	+1
20	30	12	33	13	-1
45	25	9	30	8	+1
45	19	13	20	10	+3
45	20	13	23	12	+2

In the above experiments six pairs of kidneys were taken and washed in Locke for 20 minutes, six kidneys, one from each pair, being then removed to cyanide-Locke. These and the remaining six in ordinary Locke were washed for a further period of 20 or 45 minutes. The fluid was renewed a few times in this latter washing.

The mean difference at the end between the active and inactive kidneys is 1 mg. urea per 100 ml.

Six pairs of kidneys were selected for each experimental set and washed for 20 minutes in Locke, then three re-immersed in Locke and the companion tissues in cyanide-Locke for another 45 minutes, the conditions being therefore as before, except that the Locke for re-immersion contained no urea, and during the second period of 45 minutes was changed twice to reduce the external urea concentration to an insignificant figure. The results of these experiments are contained in Table VI. The average concentration of the active kidneys at the end of the period is 11·7 mg. per 100 ml. and that of the inactive is 10·0 mg. per 100ml. The average difference is therefore only 1·7 mg. urea per 100 ml., whereas 16 mg. would have to be accounted for. Table II also shows the results of a similar set of experiments with a 20-minute period of re-immersion instead of 45 minutes. The mean urea value for the active kidneys was 17 and for the inactive 16 mg. per 100 ml. Over the whole period of 45 minutes' re-immersion, therefore, there is—for the purpose of the present discussion—no significant difference between active and inactive kidneys.

It is certain, therefore, that the large difference between the active and inactive tissues after re-immersion in urea-Locke is not due to the mere liberation of urea held up in the tubule lumen by the action of cyanide.

A point that may be mentioned in connection with the immersions of the kidneys in Locke and cyanide-Locke is that the weights of the kidneys alter. We have already studied this in some detail, and with results of much interest to be described in a subsequent communication. Here it may be said that if the original washing in Locke has been for 30 minutes with a subsequent 30 minutes' immersion in Locke or cyanide-Locke (cyanide concentration—0·02 % KCN) the difference between the final weights of the kidneys active and inactive, is less than 5 %. (This applies to the experiments of Table IV.) When the original washing in Locke was only for 20 minutes, with subsequent 45-minute immersions in Locke or cyanide-Locke, as in the experiments of Table V, the mean difference between the active and inactive kidneys was found to be about 7 %. The active kidneys summarised in Table V lost 10 % of their weight during the 45-minute re-immersion and the inactive lost 3 %. These changes in volume however will not influence the nature of the results obtained, since it is concentration equilibria we are dealing with and these are nearly reached at 45 minutes. If however the volume changes are reckoned as exercising the maximum conceivable effect, in a manner of argument opposing the above, then if we calculate the final concentrations of the kidneys in terms of the weights before instead of after immersion in the urea-Locke or urea-cyanide-Locke, the average concentration for the active kidneys of Table V becomes 46 mg. per 100 ml. and for the inactive 35 mg. (the external fluid having a urea concentration of 40 mg. per 100 ml.).

This, therefore, could not affect the argument, the inactive kidney having still only 76 % of the urea content of the active, the latter being 15 % more concentrated in urea than the external fluid—and this after attaining to less than 80 % of the final equilibrium value.

Concerning the technical side of the analyses the opinion may be entertained that since differences between urease and xanthydrol determinations on kidney suspensions have been recorded in the literature, urea analyses of the frog's kidney are uncertain. The reason for such differences has been demonstrated above in the section on methods. The following facts may also be noted.

(1) From the Locke fluid in which fresh kidneys are immersed 95 % of the total urea originally present—as determined from companion tissues—can be recovered after 3 hours, and this amount corresponds to 97 % of the theoretically diffusing quantity. The remaining 3 % may even be accounted for by the swelling of the tissues after this period.

(2) The differences in urea concentration of the active and inactive kidneys, on which the proof turns, can be shown either from unboiled suspensions, boiled suspensions, or by xanthydrol analyses. There is no difference in the figures obtained by us using the xanthydrol method with unboiled suspension, or the urease method with boiled suspension. A slight difference existed between the absolute urea concentration as determined from boiled and unboiled suspensions with our method of analysis by urease. This difference was due to the inclusion of some "extra" ammonia formed by the urease extract on the unboiled suspension, already considered in detail in the first section.

The amount of this "extra" ammonia could be considerably increased by prolonging the time of incubation of the suspension with the urease extract and also by increasing the concentration of the extract itself.

From the existence of this "extra" ammonia Rehberg has even invented the conception that one moiety of urea in the frog's kidney exists in a freely diffusible form—such as was given by the xanthydrol measurements—and another as "bound" urea which could be slowly acted upon by the ferment urease and in quantity corresponding to this "extra" ammonia.

The freely diffusible form of urea was present—according to Rehberg—in quantities little if at all greater than that of the fluid with which he perfused the kidney. The truth is however that the urea in the blood of the normal frog is only 60–70 % of the true and freely diffusible urea of the kidney, and this is so whether we analyse the urea by the xanthydrol method or by the urease method—employing the latter correctly—or whether in fact we determine the urea which has freely diffused into an external Locke solution.

Not only does this relationship of blood urea to renal urea normally exist, but if we isolate the kidney and wash it so that its concentration is well below the normal blood level and then re-immerse it in Locke solution containing urea of similar or lower concentration to that of the blood, the concentration relationships of blood and renal tissue are re-established, the urea-Locke solution in this case replacing the plasma.

The concentration of urea from an external urea-Locke being demonstrated, the question arises, where is the urea stored? Does a gradient exist across the tubule cells reaching the maximum concentration in the urine or does all the stored urea pass into the urine, the concentration of the cells being that of a passive equilibrium with the external fluid? The average urea figures found for the winter frogs was 55 mg. per 100 ml. blood and 82 mg. per 100 ml. kidney. If we assume that the urea in excess of the blood is all present in the urine, and that the urine occupies—as a maximum value—5 % of the whole tissue, then the urea in the urine must be 11 times more concentrated than the blood, which is a not improbable figure for the urine concentration when this is slowly excreted. It is possible, therefore, that all the urea concentrated from the external fluid exists in the urine. This is in fact a point of view we had previously taken in relation to substances actively secreted, and that there was no evident reason for the supposition that such substances must be concentrated within the cells, though this possibility must be allowed pending further proof.

In conclusion it may be noted that whereas in the present communication we have shown that for a secreted substance the active isolated frog's kidney comes into equilibrium with a much higher concentration than the inactive (and higher than the external fluid), the reverse effect was demonstrated for an absorbed substance. The absorbed substance in question is sodium sulphate, which in about nine cases out of ten appears in the urine of the frog (*Rana temporaria*) with only one-third or less the plasma concentration.

SUMMARY.

1. The concentration of urea from an external urea-Locke solution by the active isolated kidney of the frog has been definitely proved.

2. The proof consists in washing pairs of fresh kidneys in Locke for 20 minutes, retaining one member of each pair for a grouped analysis, the companion tissues being immersed for 45 minutes in urea-Locke, containing 40 mg. urea per 100 ml., and subsequently analysed. After the initial washing the mean urea concentration was 34 mg. per 100 ml. and after re-immersion in urea-Locke it rose to 51 mg. urea per 100 ml. When the re-immersion was made in urea-cyanide-Locke—containing also 40 mg. urea per 100 ml.—the concentration found was 35·6 mg. urea per 100 ml.

A large number of similar experiments were also carried out in which the external concentrations *etc.* were varied. The results in all cases supported the above findings.

3. When the re-immersions after the initial washing were made for 45 minutes in Locke or cyanide-Locke containing no urea, the mean difference of urea content between the active and inactive tissues did not exceed 2 mg. urea per 100 ml.

4. Changes in the volume of active and inactive (cyanide) kidneys under the conditions examined have no appreciable effect on a consideration of the results.

5. Xanthydrol determinations of the urea in the frog's kidney gave identical results with the urease method used. This latter was subjected to a detailed examination in so far as it could have any bearing on the results obtained.

REFERENCES.

Beattie (1928). *Biochem. J.* **22**, 711.
Conway (1933). *Biochem. J.* **27**, 430.
—— (1934). *Biochem. J.* **28**, 283.
—— and Byrne (1933). *Biochem. J.* **27**, 419.
—— and Kane (1934). *Biochem. J.* **28**, 1769.
Rehberg, Brandt and Clem (1932). *Pfluger's Arch.* **230**, 697.

CLXXVI. STUDIES ON THE ANAEROBIC DECOMPOSITION OF PLANT MATERIALS.

IV. THE DECOMPOSITION OF PLANT SUBSTANCES OF VARYING COMPOSITION.

By CADAMBI NARASIMHA ACHARYA.

From The Fermentation Department, Rothamsted Experimental Station, Harpenden, Herts.

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The course of anaerobic decomposition of rice straw in relation to losses of straw constituents, products formed and nitrogen transformations, has been examined in some detail in earlier communications [Acharya, 1935, 1, 2, 3]. The present paper reports the results of the anaerobic digestion of oat, wheat and barley straws, bracken leaves, young grass (lawn mowings) and rape seed cake in comparison with the decomposition of rice straw.

In one set of experiments 20 g. portions of the materials were digested without the addition of nitrogen, but with 3 g. $KHCO_3$ added to neutralise the acids formed. Wherever this amount of bicarbonate was not enough to keep the reaction alkaline, and as a result stoppage of gas evolution occurred, additions of NaOH were made from time to time. In a parallel set of experiments, ammonium carbonate equivalent to 1 % N on the material taken was added. The other experimental details and analytical methods adopted were the same as those previously described [1935, 1].

Besides the above, a few digestions were also made of the cellulose, hemicellulose and lignin fractions isolated from oat straw.

The results of anaerobic decomposition, as shown by losses of plant constituents, were also compared with the losses brought about by 3 different types of acid treatment: (1) aqueous acid, 5 % H_2SO_4; (2) alcoholic acid, a solution of

Table I. *Analysis of materials used for anaerobic digestion.*

Weights in g. per 100 g. of original material.

Constituents	Rice straw	Oat straw	Wheat straw	Barley straw	Bracken leaves	Grass (lawn mowings)	Rape seed cake
1. Cellulose (deducting xylan contained)	32·82	33·70	35·57	36·37	17·98	24·08	15·52
2. Xylan associated with cellulose	11·73	15·72	16·71	15·03	3·43	7·29	2·44
3. Xylan not associated with cellulose	9·31	8·92	8·48	7·81	1·49	3·01	4·16
4. Lignin (direct)	10·67	16·70	17·04	16·08	30·68	12·13	8·46
5. Protein	2·88	2·51	2·31	3·56	11·67	22·90	35·30
6. Ash	17·50	6·60	5·62	4·59	5·05	10·74	8·34
7. Alcohol-benzene extractives	3·05	5·88	3·52	5·47	17·50	9·54	12·01
8. Moisture	8·40	7·51	8·04	10·02	10·10	9·89	11·16
Total	96·36	97·54	97·29	98·93	97·90	99·58	97·39
9. Lignin (after hydrolysis)	9·12	13·31	12·60	11·88	21·72	10·46	7·47
10. Ash-free organic matter soluble in water	10·07	9·75	7·62	9·97	24·83	18·01	20·46
11. Dry matter less ash	74·10	85·89	86·34	85·39	85·55	74·37	80·50

5 % HCl in 95 % alcohol; and (3) an oxidising acid agent, 2 % and 5 % HNO_3. The aim of the comparison was to examine whether any of the treatments could be used as a rapid method for forecasting the degree of anaerobic digestion and the probable amount of acid or gas obtainable as a result of such digestion; ordinarily several months elapse before gas evolution stops.

Data for the composition of the seven materials under examination, given in Tables I and II, show that whilst rice, oat, wheat and barley straws resemble one another in general composition, bracken leaves, grass and rape cake differ from these and among themselves. Bracken leaves are poor in hemicelluloses (as measured by furfuraldehyde yield) and cellulose, but rich in lignin and fairly rich in protein. Grass contains a good proportion of protein and cellulose, while rape seed cake is poor in hemicelluloses but rich in proteins.

The losses of constituents as a result of anaerobic digestion are given in Table II and the products formed in Table III.

1. *Losses of constituents.*

It is noteworthy that a considerable proportion of the total decomposition can be brought about without the addition of nitrogen if the p_H and other factors influencing anaerobic digestion [Acharya, 1935, 2] are maintained at the optimum. Still, the addition of nitrogen in the form of ammonium carbonate

Table II. *Losses of constituents.*

Weights in g. per 100 g. of original material.

	Loss by fermenting for 6 months		Loss by boiling for 1 hour with			
Constituents	Without N addition	With N added	5 % H_2SO_4	5 % alc. HCl	2 % HNO_3	5 % HNO_3
	1. Rice straw.					
Dry residue less ash	31·41	35·24	29·46	27·12	34·55	37·44
Cellulose (including xylan)	19·18	21·26	15·74	10·47	14·47	11·59
Xylan in cellulose	5·72	6·36	7·75	8·36	8·65	9·08
Xylan not in cellulose	6·22	6·43	8·12	1·81	7·18	7·84
Total xylans	11·94	12·79	15·87	10·17	15·83	16·92
Lignin (direct)	2·92	3·04	1·55	3·24	5·68	7·02
Lignin (after hydrolysis)	2·51	2·60	—	—	—	—
Protein	1·02	0·87	1·46	1·42	1·34	2·14
	2. Oat straw.					
Dry residue less ash	37·79	41·14	34·12	35·14	39·85	45·53
Cellulose (including xylan)	20·62	23·96	16·39	12·85	12·94	12·18
Xylan in cellulose	6·92	8·67	9·68	9·83	10·75	11·73
Xylan not in cellulose	5·52	5·42	8·81	2·38	7·07	8·90
Total xylans	12·44	14·09	18·49	12·21	17·82	20·63
Lignin (direct)	4·55	5·04	3·39	5·46	12·11	14·37
Lignin (after hydrolysis)	3·11	3·51	—	—	—	—
Protein	1·11	1·01	1·53	1·54	Nil	1·08
	3. Wheat straw.					
Dry residue less ash	29·12	36·84	33·80	37·80	39·78	44·89
Cellulose (including xylan)	16·44	20·58	16·63	14·78	15·11	17·27
Xylan in cellulose	5·31	6·91	10·87	10·32	11·56	12·43
Xylan not in cellulose	5·71	5·88	7·25	3·16	7·58	8·24
Total xylans	11·02	12·79	18·12	13·48	19·14	20·67
Lignin (direct)	3·06	4·04	4·44	6·26	12·43	14·96
Lignin (after hydrolysis)	1·94	2·90	—	—	—	—
Protein	0·91	0·51	1·31	1·21	Nil	0·84

Table II (*cont.*).

	Loss by fermenting for 6 months		Loss by boiling for 1 hour with			
Constituents	Without N addition	With N added	5% H_2SO_4	5% alc. HCl	2% HNO_3	5% HNO_3
4. Barley straw.						
Dry residue less ash	35·12	40·06	36·19	35·35	40·41	45·64
Cellulose (including xylan)	23·20	26·54	18·68	15·79	15·36	16·39
Xylan in cellulose	6·97	7·77	10·22	10·31	10·37	11·63
Xylan not in cellulose	4·21	4·69	5·92	2·68	6·44	6·93
Total xylans	11·18	12·46	16·14	12·99	16·81	18·56
Lignin (direct)	3·10	3·27	4·20	5·16	11·11	12·85
Lignin (after hydrolysis)	1·38	1·93	—	—	—	—
Protein	1·91	1·41	2·13	2·20	0·58	1·60
5. Bracken leaves.						
Dry residue less ash	29·10	29·80	35·87	44·30	36·94	57·36
Cellulose (including xylan)	4·52	4·84	4·49	5·14	3·40	5·74
Xylan in cellulose	0·98	1·06	2·06	1·64	1·45	2·35
Xylan not in cellulose	0·60	0·96	1·06	0·12	0·88	0·74
Total xylans	1·58	2·02	3·12	1·76	2·33	3·09
Lignin (direct)	7·78	7·82	8·96	12·82	5·68	23·02
Lignin (after hydrolysis)	0·84	0·88	—	—	—	—
Protein	1·25	0·88	5·28	5·14	2·19	6·09
6. Grass (lawn mowings).						
Dry residue less ash	45·52	46·20	40·41	38·37	38·65	47·18
Cellulose (including xylan)	20·85	21·12	12·13	11·60	11·21	10·74
Xylan in cellulose	4·77	4·94	4·82	5·70	5·93	6·33
Xylan not in cellulose	1·85	2·20	2·42	0·07	2·06	2·12
Total xylans	6·62	7·14	7·24	5·77	7·99	8·45
Lignin (direct)	1·63	1·92	1·67	3·64	4·66	6·86
Lignin (after hydrolysis)	1·06	1·23	—	—	—	—
Protein	16·58	15·42	15·95	12·64	13·16	16·74
7. Rape seed cake.						
Dry residue less ash	55·02	55·40	53·60	42·07	49·15	59·50
Cellulose (including xylan)	13·72	13·91	9·65	7·22	6·41	7·26
Xylan in cellulose	1·74	1·89	1·79	1·96	1·90	2·02
Xylan not in cellulose	3·08	3·06	3·03	1·85	2·89	2·63
Total xylans	4·82	4·95	4·82	3·81	4·79	4·65
Lignin (direct)	0·60	0·62	0·99	2·22	1·37	4·55
Lignin (after hydrolysis)	0·56	0·56	—	—	—	—
Protein	30·85	29·75	28·73	19·93	24·20	27·92

stimulates the decomposition, as measured by losses of plant constituents and by-products formed, in the case of materials like straws poor in nitrogen. The effect of addition of nitrogen, however, is much less than that noted for aerobic decomposition [Hutchinson and Richards, 1921].

The degree of decomposition is approximately the same in the case of the 4 straws, about 45–50 % of cellulose, 55–60 % of hemicellulose and 25–30 % of lignin, calculated on the amounts originally present, being destroyed. With bracken, only 22 % of cellulose and 41 % of hemicellulose are decomposed and the destruction of protein is also low. This partial digestion is associated with the high lignin content of the material which, it is suggested, prejudicially affects the decomposition of the other constituents. Levine *et al.* [1935] recently found that additions of lignin to actively fermenting corn stalk flour or packing house (anaerobic) sludge inhibited gas formation, attributing this to chemical combination of lignin with the material under decomposition and formation of complexes resistant to microbial decomposition. In grass and rape seed cake cellulose and hemicelluloses suffer considerable loss; the decomposition of the proteins is also high. This rapid destruction is associated with the comparatively low content of lignin in the two materials.

2. *Products formed.*

The general nature of the by-products is the same in the seven cases, CO_2, CH_4, acetic and butyric acids being the chief substances formed. The relative amounts of acids and gases present at the end of 6 months vary, however, with the different materials, probably owing to the differing relative rates of the two stages of anaerobic decomposition, *viz.* acid formation and gas production [Acharya, 1935, 1, 2]. The gas yield is very low with rape seed cake, on account of the rapid accumulation of acids and the lack of enough $KHCO_3$ to maintain the p_H in the range favourable for gas formation.

Table III. *Products formed by anaerobic fermentation for 6 months*

Per 100 g. of material	Volume of CO_2 (corrected for carbonates) ml.	Volume of CH_4 evolved ml.	Wt. of CO_2 g.	Wt. of CH_4 g.	Acetic acid present g.	Butyric acid present g.	Total products g.
1. Rice straw, without N addition	8010	8660	15·84	6·21	2·46	0·82	25·33
Rice straw, with N addition	10870	10570	21·49	7·58	1·03	0·27	30·37
2. Oat straw, without N addition	7040	7720	13·92	5·54	7·57	1·52	28·55
Oat straw with N addition	6050	6960	11·96	4·99	11·43	4·13	32·51
3. Wheat straw, without N addition	4010	4960	7·93	3·56	10·14	3·52	25·15
Wheat straw, with N addition	9420	10150	18·62	7·27	4·29	1·63	31·81
4. Barley straw, without N addition	5240	6120	10·36	4·39	7·36	3·08	25·19
Barley straw, with N addition	7110	7980	14·06	5·72	7·22	2·52	29·52
5. Bracken leaves, without N addition	4850	6240	9·59	4·47	4·28	4·24	22·58
Bracken leaves, with N addition	5120	5910	10·13	4·23	6·23	2·83	23·42
6. Grass (lawn mowings), without N addition	9220	13240	18·23	9·49	2·52	4·26	34·50
Grass (lawn mowings), with N addition	9670	13720	19·12	9·83	1·32	4·86	35·13
7. Rape seed cake, without N addition	1920	3075	3·80	2·20	16·85	18·05	40·90
Rape seed cake, with N addition	1630	2880	3·22	2·07	17·57	18·55	41·41

The largest amount of products per 100 g. of material is obtained from rape seed cake (41 g.) followed by grass (35 g.), oat straw (33 g.), wheat straw (32 g.), rice straw (30 g.), barley straw (29 g.) and bracken (23 g.).

Whereas with straws the relative proportions of CO_2 and CH_4 evolved (after correcting CO_2 for the fraction evolved from carbonates) are of the same order, in the case of grass and rape cake the amount of CH_4 formed is much higher than that of CO_2. This difference is associated with a relatively low amount of butyric acid present in the former group and higher amounts in the latter. The bulk of the butyric acid seems to originate from the protein fraction in the case of rape seed cake, for the amount present cannot be accounted for by the losses of other constituents. It has already been noted [1935, 2] that when proteins, such as caseinogen, blood albumin and egg albumin, were added to decomposing rice straw, the proportion of butyric acid formed showed a rapid rise.

The increase in the amount of CH_4 formed and its relationship to the CO_2 produced and the butyric acid present lend support to the view advanced by Symons and Buswell [1933] that anaerobic digestion consists of a series of oxidation-reduction reactions in which water plays an important part.

3. *Nitrogen transformations.*

The nitrogen transformations during anaerobic digestion of the different materials are shown in Table IV. Positive values for nitrogen factor and nitrogen equivalent carry meanings assigned to them by Rege [1927] and Richards and Norman [1931], while negative values represent the net amount of nitrogen mineralised or the proportion of original protein reduced to ammonia at the end of the decomposition.

Table IV. *Nitrogen transformations in anaerobic decomposition.*

		After fermentation for 6 months				
Plant material	N in 100 g. of original material g.	Protein-N in residue g.	Protein-N in aqueous extract g.	Total protein-N g.	N immobilised or N factor g.	Nitrogen equivalent g.
1. Rice straw, without N addition	0·461	0·297	0·129	0·426	−0·035	−0·112
Rice straw, with N addition	0·461	0·323	0·207	0·530	+0·069	+0·196
2. Oat straw, without N addition	0·401	0·205	0·163	0·368	−0·033	−0·088
Oat straw, with N addition	0·401	0·237	0·305	0·542	+0·141	+0·343
3. Wheat straw, without N addition	0·369	0·225	0·095	0·320	−0·049	−0·168
Wheat straw, with N addition	0·369	0·305	0·230	0·535	+0·166	+0·451
4. Barley straw, without N addition	0·570	0·265	0·225	0·490	−0·080	−0·228
Barley straw, with N addition	0·570	0·344	0·420	0·764	+0·194	+0·485
5. Bracken leaves, without N addition	1·867	1·665	0·125	1·790	−0·077	−0·265
Bracken leaves, with N addition	1·867	1·727	0·155	1·882	+0·015	+0·050
6. Grass, without N addition	3·664	1·011	0·425	1·436	−2·228	−4·895
Grass, with N addition	3·664	1·197	0·505	1·702	−1·962	−4·247
7. Rape seed cake, without N addition	5·646	0·712	0·675	1·387	−4·259	−7·739
Rape seed cake, with N addition	5·646	0·888	0·510	1·398	−4·248	−7·668

The data show that when no additional nitrogen is added, about 10 % of the original protein is converted into ammonia with the straws, while the proportion is much higher with grass (60·8 %) and rape seed cake (75·4 %). Though bracken contains a comparatively high percentage of nitrogen (1·867 %) only 4·1 % of it is converted into ammonia. This may be due either to the difference in the nature of the proteins present or to the protective action exercised by the high lignin content in the bracken.

The distinct, though slight, stimulating action exercised by addition of available nitrogen on the degree of decomposition and the amount of decomposition products, has been referred to above. Nitrogen supply appears also to modify the course of nitrogen transformations in the anaerobic system, as shown by the values for nitrogen factor and equivalent. With materials poor in nitrogen, *e.g.* straws, these values are negative in the absence of added nitrogen and positive in its presence. The positive values represent a net gain of synthesised protein during the period of digestion. They are, however, much lower than the figures given for aerobic decomposition [Richards and Norman, 1931]. With materials rich in anaerobically available nitrogen, *e.g.* rape seed cake or grass, the nitrogen factor and equivalent give high negative values, both in presence and absence of added nitrogen, showing that the net result in either case at the end of the digestion is the reduction of a considerable portion of the original protein to ammonia. Bracken, however, differs in showing a low negative value in the absence of nitrogen addition and a small positive value in its presence, thus resembling the straws poor in nitrogen. This behaviour of bracken could be explained if it be assumed that for purposes of anaerobic digestion the protein is not available.

4. *Comparison of the losses during anaerobic digestion with those during acid hydrolysis.*

10 g. portions of the materials were boiled for one hour with 200 ml. of (1) aqueous 5 % H_2SO_4; (2) a solution of 5 % HCl in 95 % alcohol; (3) aqueous 2 % HNO_3; (4) aqueous 5 % HNO_3. The residues were weighed and analysed for cellulose, furfuraldehyde yield, lignin and protein. The losses of constituents by the different treatments, calculated on 100 g. of original material, are presented in Table II.

It will be noted that none of the acid treatments gives the same results as those obtained by anaerobic digestion, the difference being most marked in the case of cellulose. A considerable proportion of cellulose, varying from 40–60 % of the amount originally present, is lost by anaerobic digestion, whilst the losses by acid treatment amount to 25–30 % only. The higher concentration of nitric acid (5 %) was used to oxidise the cellulose, if possible, and so secure a greater loss; but the attempt was unsuccessful.

A second difference between the two kinds of decomposition lies in the relative proportions of cellulose to xylan, associated with cellulose, left after decomposition. In anaerobic fermentation the amount of cellulose which disappears is roughly proportional to the loss of xylan associated with it. But acid treatments disturb this ratio by removing a much higher proportion of the associated xylan than of cellulose.

The differential removal of the straw constituents by the four acid treatments is worth noting. While 5 % H_2SO_4, 2 % HNO_3 and 5 % HNO_3 remove most of the xylan not associated with cellulose and also a large portion of the xylan so associated, 5 % alcoholic HCl acts mostly on the xylan fraction associated with cellulose and much less on the fraction not so associated.

Nitric acid at 2 % as well as 5 % concentration oxidises and dissolves large proportions of the lignin originally present, ranging from 60 to 80 %, and would therefore be unsuitable for purposes of comparison with anaerobic treatment, where the losses average about 25–30 % only. With bracken leaves the loss of lignin is low with 2 % HNO_3; this is associated with a smaller loss of protein as compared with the other acid treatments. It is probable that a portion of the protein might have been oxidised by the nitric acid with the formation of "apparent" lignin [Norman and Jenkins, 1934] which may mask the loss of lignin originally present.

With straws the losses in protein are higher during acid treatment than during anaerobic digestion, but there is good agreement in the case of materials richer in nitrogen, *e.g.* grass and rape seed cake. Bracken, however, is an exception since its protein is less soluble under anaerobic conditions than with acid treatment. This low solubility under biological action may be due, as already observed, to the protective action of lignin. The nitrogen losses on treatment with nitric acid are irregular, owing possibly to the nitrating action of the acid on the constituents.

Of the four acid treatments tried, that with 5 % H_2SO_4 seems to come closer to the results of anaerobic digestion than the others. The attack on cellulose is no doubt distinctly lower than occurs in the biological action, but this is to a certain extent made up by the greater attack on the xylans. Considering the widely varying composition of the straws on the one hand and of bracken, grass and rape cake on the other, the agreement may be considered satisfactory. Bearing in mind the remarks made above in regard to the attack on cellulose and xylans, the method can be put forward as one which could rapidly give some idea of the anaerobic decomposability of a plant material and the approximate amount of products, either acid or gas, which could be obtained from it. More work with a larger variety of substances is necessary before any definite recommendations in the matter can be made.

5. *Decomposition of constituents isolated from oat straw.*

A comparative study of the anaerobic decomposition of the different constituents of oat straw was made using preparations of lignin, hemicellulose and cellulose isolated from it. The sample of lignin was obtained by treatment with 72 % H_2SO_4, of hemicellulose by boiling with 4 % NaOH and subsequent precipitation with acetic acid and alcohol, and of straw cellulose by the method of Norman and Jenkins [1933]. A portion of the cellulose was treated with boiling 5 % H_2SO_4 for one hour to remove a part of the xylan contained and was also fermented for purposes of comparison. The sample of crude hemicellulose gave a furfuraldehyde yield equivalent to 76·8 % xylan. The sample of oat cellulose and that treated with acid contained 30·81 and 19·64 % respectively of xylan.

To 5 g. portions of the substances 2 g. $KHCO_3$, 2 g. NH_4HCO_3, 0·2 g. K_2HPO_4, 0·05 g. $MgSO_4,7H_2O$, 10 ml. of an extract from a rice soil, 10 g. of asbestos to allow for the surface development of a bacterial film and 100 ml. of water were added, the other experimental details being as usual. Gas evolution began earliest with the hemicellulose preparation, but in all cases there was a rapid accumulation of acids with consequent lowering of p_H and fall in gas yield.

The data given in Table V show that "acid" lignin does not undergo any appreciable decomposition and yields no gases or acids. Hemicellulose is more rapidly decomposed than cellulose as shown by the larger yield of gases and smaller accumulation of acids at the end of 6 months. There is no appreciable difference between the rates of fermentation of oat cellulose and that treated with 5 % H_2SO_4 for one hour.

Table V. *Decomposition of constituents isolated from oat straw.*

g. per 100 g. after 6 months' fermentation.

	Acid lignin	Crude hemi-cellulose	Oat cellulose	Purified oat cellulose
Unfermented insoluble residue	97·2	8·2	17·2	18·1
Volume of CO_2 (corrected) for carbonates (ml.)	Nil	11,120	3,820	3,340
Volume of CH_4 evolved (ml.)	,,	12,830	4,410	3,860
Weight of CO_2	,,	21·99	7·55	6·60
Weight of CH_4	,,	9·20	3·16	2·77
Acetic acid present	,,	31·32	50·56	53·58
Butyric acid present	,,	8·27	13·34	14·70
Total products	,,	70·78	74·61	77·65
Nitrogen immobilised or nitrogen factor	,,	0·280	0·320	0·340
Nitrogen equivalent	,,	0·305	0·387	0·415

It is interesting to note that though the amounts of gases and acids formed vary considerably between the hemicellulose and cellulose preparations used in the present experiment, the proportions of acetic to butyric acids and of CO_2 to CH_4 in the two cases appear to be similar. Thus with both the ratio of acetic to butyric is roughly 4 : 1 and that of CO_2 to CH_4 by volume, roughly 7 : 8. The bearing of this relationship on the decomposition of straws, where cellulose and hemicellulose are the chief constituents decomposed, will be discussed later.

The hemicellulose and cellulose preparations also show similar values for nitrogen factor and nitrogen equivalent. The values for nitrogen factor are higher than with straws, probably owing to more complete utilisation of the extracted constituents than of the plant material as a whole. The nitrogen equivalent values, representing the nitrogen immobilised per 100 g. of material actually decomposed, are of the same order for straw and for its isolated constituents.

DISCUSSION.

In the aerobic decomposition of plant materials it has been observed that lignin inhibits the decomposition of pentosans and cellulose; Rege [1927] proposed a pentosan/lignin factor as a means of predicting decomposability of substances [*cf.* Norman, 1929]. The present experiments indicate that lignin may act as a still more powerful inhibitor in anaerobic decomposition, not only of hemicelluloses and cellulose but also of proteins as well.

In all the plant residues examined there are appreciable fractions of cellulose, hemicelluloses and proteins which are left undecomposed. This may be due either to the inhibitory action of lignin or to the above fractions being encrusted on the cell wall so as not to be easily accessible for microbial attack. It should be noted that a large proportion of the cellulose and appreciable proportions of hemicelluloses and proteins remain also unattacked by weak acids.

The experiments on the nitrogen relationships confirm what has already been described for rice straw regarding the low nitrogen requirements of anaerobic digestion and the absence of any considerable synthesis of proteins from ammonia.

The products obtained by anaerobic decomposition of the cellulose and hemicellulose fractions isolated from oat straw show an interesting similarity both as to the qualitative and quantitative nature of the components. Thus the proportion of acetic to butyric acids is roughly 4 : 1. The approximate constancy of this proportion leads us to expect a similar ratio with straws, where loss of cellulose and hemicellulose accounts for the greater part of the organic matter which disappears. It is proposed to extend this work to an examination of the products obtained by anaerobic digestion, under conditions favouring acid and gas production respectively, of a large variety of cellulose, hemicellulose, protein and other fractions isolated from different plant materials and to compare the data obtained with those for plant materials pretreated so as to remove most of certain constituents.

The absence of gas or acid production from "acid" lignin confirms the work of others [Levine *et al.*, 1935], but it must be noted that the susceptibility of lignin in the natural state to anaerobic attack may be different from that of the fraction isolated by acid or alkali treatment. Recently Levine *et al.* [1935] have suggested that much of the loss of lignin hitherto reported in anaerobic digestion [*cf.* Boruff and Buswell, 1930] may be due to the mechanical solution of lignin rather than to its chemical decomposition. But in the experiment on "acid" lignin there was an almost complete recovery of added lignin, indicating that at the p_H maintained the solution of "acid" lignin was not appreciable. However, further work is necessary before this point can be satisfactorily settled.

The results of acid treatment show marked differences from those obtained by anaerobic digestion. During the course of decomposition by micro-organisms, cellulose is destroyed more rapidly than with mineral acids and the losses in cellulose keep pace with those in the xylan fraction associated with it. The preferential extraction by alcoholic acid of the xylan fraction associated with cellulose is also noteworthy. The use of an alcoholic solution of sulphuric acid and a correction factor applied for the cellulose actually decomposed, might perhaps give results which approximate to the losses found under anaerobic conditions.

SUMMARY.

1. The anaerobic decomposition of oat, wheat, rice and barley straws, bracken leaves, young grass (lawn mowings) and rape seed cake has been studied with reference to losses of constituents, products formed and nitrogen transformations.

2. A good proportion of the total decomposition could be brought about by digesting the materials without the addition of nitrogen, under conditions of optimum temperature and reaction. With materials poor in available nitrogen, however, *e.g.* straws, the addition of nitrogen exerts a distinct, though slight, stimulating effect.

3. The chief products obtained are the same in all the cases: *viz.* acetic and butyric acids, CO_2 and CH_4.

4. Whilst the straws yield a large amount of acetic and small amounts of butyric acid, the reverse is the case with materials rich in available protein, *e.g.* grass and cake. The $CO_2 : CH_4$ ratio is roughly unity in the former case, while in the latter the proportion of CH_4 is much higher.

5. The losses of plant constituents by anaerobic fermentation have been compared with those brought about by boiling for one hour with (1) aqueous 5 % H_2SO_4; (2) alcoholic 5 % HCl; (3) aqueous 2 % and 5 % HNO_3. The cellulose fraction destroyed by acid treatment was smaller and the hemicelluloses decomposed greater than by biological agency. It is suggested that the losses on treatment with 5 % H_2SO_4 for one hour, with a correction for the cellulose decomposed, might be used to forecast the anaerobic decomposability of a plant material and the probable yield of acid and gaseous products.

6. The decomposition of lignin, hemicellulose and cellulose fractions isolated from oat straw has been studied. Lignin is not fermented. Hemicellulose and cellulose fractions are fermented, the former more readily than the latter, yielding acetic and butyric acids in the proportion of 4 : 1 (approx.) and CO_2 and CH_4 in the ratio of 7 : 8 (approx.).

7. The presence of a high lignin content markedly inhibits the anaerobic decomposition of protein and other constituents.

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REFERENCES.

Acharya (1935, 1). *Biochem. J.* **29**, 528.
—— (1935, 2). *Biochem. J.* **29**, 953.
—— (1935, 3). *Biochem. J.* **29**, 1116.
Boruff and Buswell (1930). *Ind. Eng. Chem.* **22**, 931.
Hutchinson and Richards (1921). *J. Min. Agric., London*, **28**, 398.
Levine, Nelson, Anderson and Jacobs (1935). *Ind. Eng. Chem.* **27**, 195.
Norman (1929). *Biochem. J.* **23**, 1367.
—— and Jenkins (1933). *Biochem. J.* **27**, 818.
—— —— (1934). *Biochem. J.* **28**, 2147, 2160.
Rege (1927). *Ann. Appl. Biol.* **14**, 1.
Richards and Norman (1931). *Biochem. J.* **25**, 1769.
Symons and Buswell (1933). *J. Amer. Chem. Soc.* **55**, 2028.

CLXXVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XIII. NEUTRAL SALT EFFECTS.

BY FRANK DICKENS AND GUY DRUMMOND GREVILLE[1].

From the Cancer Research Laboratory, North of England Council of the British Empire Cancer Campaign, Royal Victoria Infirmary, Newcastle-upon-Tyne, and the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

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THE study of the metabolism of surviving tissue in the form of slices, that is, in the form in which least damage has occurred, is being pursued by many workers as a method of elucidating the metabolism of the living body cells. This method, whilst having the great advantage of using intact cells, is limited in comparison with the analytical method in which tissue enzymes, carriers *etc.* are extracted and studied; nevertheless, much has been, and is being, learnt by making changes in the medium in which the slices are suspended, particularly by the addition of substances foreign to the body. A better understanding of the nature of the effects produced, and a correlation of the action of different substances, is however needed before theories of cell metabolism, based on these actions, can usefully be evolved.

It has recently been found that when 0·1M KCl is added to the Ringer solution in which slices of rabbit brain cortex are respiring, both the respiration and aerobic glucolysis assume values much larger than the values in pure Ringer solution [Ashford and Dixon, 1935]. This profound alteration of the metabolism is of great interest to the writers, since they have observed similar actions on various kinds of tissue by certain organic coloured substances in very low concentrations. Thus, thionine increases the respiration of tumours without changing the aerobic glucolysis [Dickens, 1934], whilst phenosafranine increases the aerobic glucolysis of brain and tumours without affecting the respiration [Dickens, 1935]. Further, dinitro-*o*-cresol simultaneously increases the respiration and aerobic glucolysis, not only in tumours [Dodds and Greville, 1934], but also in brain tissue [Greville, unpublished observations], thus producing in the latter exactly the same effect as potassium chloride. We were led, therefore, to study the potassium effect more closely in order to seek its cause; to observe the result of other alterations of the cations in the medium; and to enquire whether the potassium effect, like the action of the coloured substances, occurs with tissues other than the brain cortex.

EXPERIMENTAL METHODS AND RESULTS.

The measurements of respiration in phosphate medium and of anaerobic glucolysis in bicarbonate medium were made in the usual way [Warburg, 1926]. Simultaneous measurements of respiration and aerobic glucolysis were made sometimes by the two-vessel method [Warburg, 1924] and sometimes by the method of Dickens and Šimer [1930, 1] for the determination of R.Q. The latter, as ordinarily used, gives low values for the aerobic glucolysis when this is high compared with the respiration. If however the amount of tissue and the duration of the experiment be reduced so that the change in bound CO_2 does not exceed say 250 μl., then, although the R.Q. cannot be measured accurately, good values are obtained for the respiration and aerobic glucolysis, and the advantage of the method is retained, the actual measurement being made on one tissue slice.

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[1] Halley-Stewart Research Fellow.

Media.

Unless otherwise stated, the suspension media were the following:

	Bicarbonate medium		Phosphate medium	
	%	M	%	M
NaCl	0·70	0·120	As in bicarbonate medium	
KCl	0·018	0·0024		
$CaCl_2$	0·019	0·0017		
$MgCl_2$	0·0076	0·0008		
Glucose	0·20	0·011		
$NaHCO_3$	0·21	0·025	—	—
NaH_2PO_4	—	—	0·040	0·0033
Na_2HPO_4	—	—	0·21	0·015

The salt concentrations are those used by Warburg [1923], except that a small quantity of $MgCl_2$ is introduced (*cf.* Tyrode's solution).

Effect of high concentrations of neutral salts on the metabolism of brain tissue.

1. *Rat brain.* Ashford and Dixon used rabbit brain. We have found that addition of 0·1 M KCl increases the respiration and aerobic glucolysis in rat brain also; and rat tissues only have been used by us.

In the experiments in phosphate medium, solid KCl was weighed into the side-bulb of the manometric vessels, and after a period of 30–40 mins., during which the oxygen uptake was noted, the KCl was washed into the medium by tipping backwards and forwards. The readings for the next 10 mins. were neglected, owing to a pressure increase on addition of the solid to the solution; thereafter a greatly increased respiration was observed. This was not however steadily maintained; often it fell off severely with time, so that 90 mins. after the addition the respiration might be little higher than that in the control where no KCl was added. For this reason most of the experiments were of short duration.

In the experiments in bicarbonate medium the solid KCl was dissolved in the medium before it was measured into the vessels. The large increase in respiration (Q_{O_2}) and aerobic glucolysis ($Q_G^{O_2}$) which occurs under these conditions is seen in Exp. 4 of Table I.

2. *Effect of substrate.* 0·1 M KCl still produces a large increase in respiration when the glucose in the medium is replaced by an equal concentration of lactate, pyruvate or fructose (Exps. 1, 2, 3, Table I). It follows that the increased respiration in glucose is not a result of increased lactic acid production.

There is no increase in the absence of added substrate (Exp. 5); and when the tissue is removed from a glucose-containing solution in which an increased metabolism has been produced by 0·1 M KCl, the solution itself has a negligible oxygen uptake. Hence 0·1 M KCl produces a general stimulation of carbo hydrate respiration, and the increased oxygen uptake is apparently not due to oxidation of cell material, either inside or outside the tissue. R.Q. measurements in phosphate medium by the method of Dickens and Šimer [1930, 1] showed that the increased oxidation occurring in presence of excess KCl proceeds, like the normal glucose oxidation of brain tissue, at R.Q. unity:

Exp. 0.	*Medium.* Ordinary phosphate-Ringer solution		The same containing *M*/10 KCl	
	Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
Duration, 80 mins.	-11·2	1·02	-18·5	1·01
	-10·0	1·00	-15·3	0·99

Table I.

Exp. 1. P.* Substrate ...	Glucose	Pyruvate	Lactate	Fructose
Q_{O_2} before 0·1 *M* KCl addition (30 mins.)	-15·0	-15·0	-19·8	-13·8
Q_{O_2} after 0·1 *M* KCl addition (30 mins.)	-25·8	-21·4	-26·8	-23·6

Exp. 2. P. Substrate ...	Glucose		Lactate	
KCl addition ...	None	0·1 *M*	None	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	-11·4	-11·4	-15·0	-15·2
Q_{O_2} after KCl addition (first 30 mins.)	-13·0	-23·9	-14·5	-22·9
Q_{O_2} after KCl addition (further 60 mins.)	-14·2	-17·3	-15·0	-15·6

Exp. 3. P. Substrate ...	Glucose			Fructose		
KCl addition ...	None	0·1 *M*	0·05 *M*	None	0·1 *M*	0·05 *M*
Q_{O_2} before KCl addition (30 mins.)	-10·2	-10·2	-10·4	-10·0	-10·8	-12·2
Q_{O_2} after KCl addition (first 30 mins.)	-10·5	-19·3	-20·6	-10·1	-21·6	-24·7
Q_{O_2} after KCl addition (further 30 mins.)	-10·9	-14·4	-16·6	-10·4	-15·0	-16·6

Exp. 4. BW. Substrate: glucose.

	No KCl		0·1 *M* KCl	
	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
First 30 mins.	-10·0	+4·2	-21·0	+18·3
Second 30 mins.	-10·0	+4·7	-17·6	+15·6

Exp. 5. BW. Substrate: none added.

	No KCl		0·1 *M* KCl	
	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
First 30 mins.	-5·1	-1·0	-0·7	-0·2
Second 30 mins.	-2·1	-1·2	—	—

* Experiments done in phosphate medium denoted P. Experiments done by Warburg method (bicarbonate medium) denoted BW. Experiments done by Dickens-Šimer method (bicarbonate medium) denoted BD.

Table II.

Exp. 6. P. Substrate: glucose.

Cl addition (0·1 *M*) ...	None	Li	Na	K	Rb	Cs
Q_{O_2} before Cl addition (30 mins.)	-14·0	-12·8	-13·0	-11·4	-11·7	-11·5
Q_{O_2} after Cl addition (first 30 mins.)	-13·7	-15·9	-14·0	-23·8	-22·4	-21·6
Q_{O_2} after Cl addition (second 30 mins.)	-14·9	-12·6	-14·8	-19·5	-18·1	-17·7

(Repeated with similar result.)

Exp. 7. P. Substrate: glucose.

Cl addition (0·1 *M*) ...	K	Mg
Q_{O_2} before Cl addition (30 mins.)	-15·0	-16·5
Q_{O_2} after Cl addition (30 mins.)	-25·8	-14·0

Exp. 8. P. Substrate: glucose.

Salt added ...	None	0·07 *M* NaCl	0·09 *M* NaCl	0·07 *M* NaCNS	0·09 *M* NaCNS
Q_{O_2} before salt addition (40 mins.)	-11·5	-11·5	-11·7	-11·3	-12·4
Q_{O_2} after salt addition (40 mins.)	-11·0	-14·4	-13·3	-14·3	-15·2

3. *Effect of other cations.* Rubidium and caesium chlorides in $0{\cdot}1M$ concentration produce increases in respiration of the same order as KCl; $0{\cdot}1M$ NaCl and $0{\cdot}1M$ LiCl have small effects; $0{\cdot}1M$ $MgCl_2$ has none (Table II). Hence for the effect produced by the alkali cations, Li, Na $<$ K, Rb, Cs. It follows that the "potassium effect" is not an effect specific to the potassium ion, but rather that the increase of respiration in brain may be produced by the metals at a particular end of the lyotropic series of the cations.

Ashford and Dixon [1935] observed that $0{\cdot}1M$ KCl decreased the anaerobic glucolysis of rabbit brain: this was confirmed for rat brain; and it was found that the same concentration of NaCl had little effect:

Exp. 9. Anaerobic glucolysis.

$(0{\cdot}1M)$	Na	K
$Q_G^{N_2}$ before Cl addition (20 mins.)	$+16{\cdot}2$	$+18{\cdot}0$
$Q_G^{N_2}$ after Cl addition (30 mins.)	$+13{\cdot}6$	$+4{\cdot}0$

Influence of the constituents of the medium on the metabolism of brain.

The effects described above, increases of respiration and aerobic glucolysis, are produced by raising the proportion of one constituent of the suspension medium. Now this medium is a "balanced" salt solution, the cations being in proportions which have been found most suitable for the maintenance of physiological function. Decreasing the concentration of the other constituents might well have a similar effect to increasing the concentration of K. It has indeed been found that Ca, K and Mg, in the concentrations in which they are usually present in the suspension medium, have a profound effect on the respiration of brain.

Effect on respiration. A number of experiments in which Ca, K and Mg were left out in turn are summarised in Table III. The concentration of Na was kept constant; the table shows the effect of adding the chlorides of calcium, potassium and magnesium singly, in pairs, or altogether, in the concentrations in which they are normally present in the suspension medium (*i.e.* $0{\cdot}0024M$ K, $0{\cdot}0017M$ Ca and $0{\cdot}0008M$ Mg), to the sodium chloride-sodium phosphate mixture. Glucose was present in $0{\cdot}2\ \%$ concentration, and the Q_{O_2} values, most of which represent the mean values of several simultaneous measurements, refer to the first 30 or 40 mins. of the experiment.

Table III.

Q_{O_2} in phosphate medium.

Cations present...	Na	Na + Ca	Na + K	Na + Ca + K	Na + Ca + K + Mg
Exp. 25	—	—	$-18{\cdot}7$	—	$-10{\cdot}4$
,, *46*	—	—	$-18{\cdot}1$	$-15{\cdot}8$	—
,, *47*	—	—	$-18{\cdot}9$	$-17{\cdot}2$	—
,, *10*	$-17{\cdot}8$	$-15{\cdot}8$	$-18{\cdot}0$	$-16{\cdot}3$	$-13{\cdot}2$
,, *48*	$-16{\cdot}9$	$-14{\cdot}1$	$-17{\cdot}7$	$-17{\cdot}2$	$-9{\cdot}0$
,, *26*	$-12{\cdot}6$	$-13{\cdot}2$	$-17{\cdot}2$	$-13{\cdot}1$	—
,, *49*	—	—	—	$-15{\cdot}2$	$-12{\cdot}4$
,, *50*	—	$-13{\cdot}5$	$-17{\cdot}0$	$-13{\cdot}1$	$-11{\cdot}6$
,, *23*	$-14{\cdot}2$	$-10{\cdot}8$	$-15{\cdot}0$	$-11{\cdot}2$	—
,, *51*	$-15{\cdot}0$	$-14{\cdot}1$	$-18{\cdot}8$	$-17{\cdot}2$	—
,, *11*	—	—	$-20{\cdot}0$	$-17{\cdot}2$	$-12{\cdot}7$
,, *52*	—	—	$-19{\cdot}3$	$-16{\cdot}0$	$-11{\cdot}5$
,, *53*	—	—	$-17{\cdot}9$	—	$-12{\cdot}6$
,, *54*	—	—	$-15{\cdot}8$	—	$-10{\cdot}6$

Some typical experiments are reported in Table IV and from these and Figs. 1 and 2 it will be seen that the respiration falls off rapidly in the absence of Ca, K and Mg. Addition of K makes the respiration steadier, but the respiration is most nearly constant when Ca, K and Mg are all present. In every experiment the respiration in the first half hour is highest when the cations present are Na and K. Addition of Ca to this mixture always lowers the respiration, and the addition of Mg to the mixture of Na, K and Ca lowers the respiration still further. This is seen best from the mean values given below.

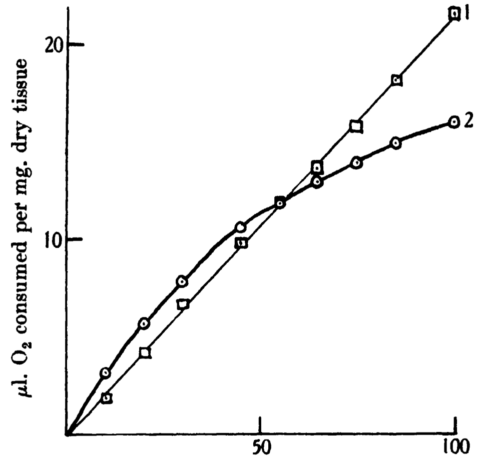

Fig. 1.

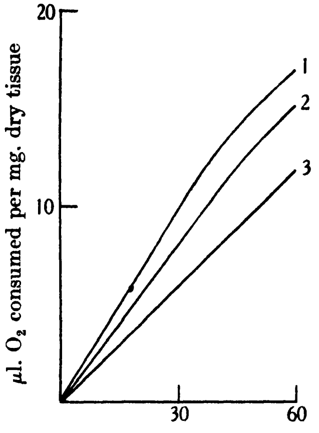

Fig. 2.

Fig. 1. Rat brain cortex. Respiration in glucose-phosphate.
1. Na + Ca + K. 2. Na.

Fig. 2. Rat brain cortex. Respiration in glucose-phosphate.
1. Na + K. 2. Na + K + Ca. 3. Na + K + Ca + Mg.

Table IV.

Exp. 10. P.	Na	Na + Ca	Na + K	Na + Ca + K	Na + Ca + K + Mg
Q_{O_2} (first 30 mins.)	−17·8	−15·8	−18·0	−16·3	−13·2
Q_{O_2} (second 30 mins.)	−11·6	−10·0	−15·4	−13·6	−12·5

Exp. 11. P.	Na + K	Na + K + Ca	Na + K + Ca + Mg	Na + K + Mg	Na + K + Mg*
Q_{O_2} (first 30 mins.)	−20·0	−17·2	−12·7	−16·2	−14·5
Q_{O_2} (second 30 mins.)	−18·0	−16·2	−12·3	−15·8	−14·3

(Experiment repeated with similar result.)

* Mg present as 0·0008 *M* $MgSO_4$.

Simultaneous measurements in all the media to be compared were not made in all the experiments of Table III. It is therefore necessary to take the media in pairs, and in reckoning the mean values for each pair, only to consider those experiments in which measurements were made simultaneously in both media. We then obtain the following mean values of Q_{O_2}:

Na+K	-18·0	Na+K	-18·0
Na+Ca+K	-15·4	Na+Ca+K+Mg	-11·5
(10 experiments)		(8 experiments)	
Na+Ca+K	-15·8	Na+K	-17·3
Na+Ca+K+Mg	-11·8	Na+Ca	-13·6
(6 experiments)		(6 experiments)	

Not much attention should be paid to the respiration figures in the medium containing Na as the only cation, owing to the rapid fall of Q_{O_2} with time (Fig. 1), but it is probable that the initial rate is very high.

The effect of Mg in lowering the respiration not only in the Na+K medium (Exp. 11), but also in the medium containing Na, K and Ca, is very striking, the concentration of Mg achieving this effect being only $8 \times 10^{-4} M$. As certain balanced solutions contain magnesium sulphate (*e.g.* artificial sea-water and the suspension medium used by Krebs and Henseleit [1932]), the effect of this salt was tried (Exp. 11). It lowers the respiration, and apparently slightly more so than an equivalent concentration of magnesium chloride.

After the completion of this work, it was reported by Chang *et al.* [1935] that the equivalence of Mg and Ca in lowering the respiration of nerve "does not extend to the grey matter of the brain, magnesium ion appearing indifferent to the respiration of this tissue despite its well-known 'anaesthetic' action". Our own experiments are so clear-cut that we can only ascribe these contradictory findings to the very different experimental conditions employed.

Effect on aerobic glucolysis. It will be remembered that 0·1 *M* KCl increases not only the respiration but also the aerobic glucolysis of brain. It might therefore be that removal of Ca from the suspension medium would also lead to an increased aerobic glucolysis, as well as an increased respiration. This is indeed so (Table V). In a solution containing Na as the only cation, the aerobic glucolysis is very high. This high acid production does not result from damage to the respiration without accompanying damage to the glucolysis (as with cyanide addition or as a result of physical damage to the tissue [Warburg, 1929]), since it appears while the respiration is higher than in the medium containing bivalent cations. Its appearance has been confirmed by the method of Dickens and Šimer (Exp. 13). Lactic acid determinations by the method of Friedemann and Graeser [1933] have shown that the acid produced is in the highest probability lactic acid.

Table V.

Exp. 12. BW.	Na		Na+Ca		Na+K		Na+Ca+K	
	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
First 30 mins.	-19·6	+28·2	-16·6	+8·1	-14·3	+8·5	-10·8	0·0
Second 30 mins.	-16·5	+17·3	-14·5	+6·2	-13·2	+4·5	-10·9	0·0
Further 20 mins.	- 9·6	+16·7	-16·1	+9·5	-10·7	+5·2	—	—

Exp. 13. BD.	Na				Na+Ca+K+Mg	
	Q_{O_2}		$Q_G^{O_2}$		Q_{O_2}	$Q_G^{O_2}$
90 mins.	-15·6	-13·6	+18·9	+18·3	-14·3	+4·2

Exp. 14. Duration 90 mins.

Medium ...	Na + Ca + K + Mg	Na	
Lactic acid at beginning (mg.)	0·12	0·13	0·11
Lactic acid at end (mg.)	0·16	0·91	0·77
Lactic acid formation expressed as $Q_G^{O_2}$	+ 1·0	+ 29·9	+ 20·2

Whilst, as we have seen, the respiration is highest in the absence of bivalent cations and the presence of K, this is not true of the aerobic glucolysis. The presence of either K or Ca in the NaCl medium lowers the aerobic glucolysis, though perhaps not so efficiently as the presence of these two ions together:

Duration 60 mins.

Medium ...	Na + Ca		Na + K		Na + Ca + K		Na only	
	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
Exp. 15	- 13·0	+ 3·8	- 17·0	+ 2·4	- 13·2	+ 0·9	—	—
Exp. 16	- 15·3	+ 7·8	- 13·2	+ 4·5	- 10·9	+ 0·0	- 13·1	+ 17·0

Reversal of the NaCl effect. To test whether the NaCl action could be reversed by the addition of Ca and K ions, the brain slices were suspended in NaCl-NaHCO$_3$-glucose solution in Warburg box-shaped vessels (two-vessel method) with side-bulbs. The latter contained amounts of isotonic solutions of KCl and CaCl$_2$ which on addition to the medium would give the usual concentrations of those salts. It was first found that the separate addition of either CaCl$_2$ or KCl to the NaCl medium caused the aerobic glucolysis immediately to fall from its high level. At the same time, however, the respiration decreased rapidly. On addition of Ca and K together the glucolysis fell to a still lower value, but the respiration was now maintained at a level only slightly below that of the control in which all three cations had been present throughout (Table VI). Hence the action of Na ion on brain is reversed by addition of the other cations of Ringer solution. The exposure of the tissue to NaCl before the addition should not exceed about 40 mins.

Table VI. *Reversal of "Na" effect.*

	Medium ...	Na		Na		Na (control)		Na + Ca + K (control)	
		Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
Exp. 17	0–20 mins.	- 24·7	+ 16·8	- 16·6	+ 12·6	- 14·2	+ 16·6	- 9·1	+ 2·1
		Add CaCl$_2$		Add KCl		↓	↓	↓	↓
	After addition	- 4·0	+ 3·6	- 1·9	+ 7·5	- 10·4	+ 16·6	- 11·1	+ 3·5

Exp. 18		Na		Na		Na + Ca + K	
		Q_{O_2}	$Q_G^{O_2}$				
	0–30 mins.	- 17·9	+ 13·9	- 13·2	+ 9·4	- 8·0	+ 0·2
		Add CaCl$_2$ + KCl				↓	↓
	After addition	- 7·9	+ 0·3	—	—	- 8·3	+ 0·2

Exp. 19		Na		Na	
	0–20 mins.	- 17·0	+ 12·1	- 16·6	+ 12·7
		Add CaCl$_2$ + KCl		↓	↓
	After addition	- 7·0	+ 0·4	- 10·4	+ 12·7

Effect on anaerobic glucolysis. We have seen that removing all the cations except Na from the suspension medium has the same effect on the aerobic glucolysis as adding 0·1 *M* KCl to the medium. Is the effect on the anaerobic glucolysis the same? Addition of 0·1 *M* KCl to the suspension medium decreases the anaerobic glucolysis of brain [Ashford and Dixon, 1935]: we find that removal of all the cations except Na lowers the anaerobic acid production in just the same way (Table VII).

Table VII.

	Medium ...	Na + Ca + K + Mg	Na
Exp. 20	$Q_G^{N_2}$ (first 30 mins.)	21·2, 21·4	9·8
	$Q_G^{N_2}$ (second 30 mins.)	19·6, 19·0	8·2
Exp. 21	$Q_G^{N_2}$ (first 30 mins.)	35·8, 22·0	13·8, 14·0
	$Q_G^{N_2}$ (second 30 mins.)	26·2, 15·8	8·0, 8·4

Influence of the constituents of the medium on the effect of high concentrations of potassium chloride.

In all experiments so far described in which an increase of brain metabolism is evoked by the addition of 0·1 *M* KCl to the medium, the tissue has been under conditions of considerable hypertonicity. We have also studied the effect of 0·1 *M* KCl in an isotonic medium, by replacing part of the NaCl with isotonic KCl to give a final K concentration of *M*/10 (Table VIII). The respiration was actually decreased in three experiments out of four, but there was a slight increase in the aerobic glucolysis (Table VIII). These results, which are in striking contrast to the effects in hypertonic solution, we ascribe to the lowering of the Na ion concentration. This view has received a certain amount of confirmation. In Exp. 24 the NaCl in the medium (which contained the usual concentrations of $CaCl_2$ and KCl but no $MgCl_2$) was replaced by glucose to give a final glucose concentration of 4·5 %. It was considered from the experiments of Loeb [1913] on the change in weight of muscle in glucose solutions that this solution should be roughly "isotonic". The addition of 0·1 *M* KCl (as solid) to this medium caused no marked increase in respiration.

Table VIII.

Exp. 22. P.	Control		0·1 *M* KCl (isotonic solution)	
	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
60 mins.	− 9·9	—	− 8·8	—
BW.				
First 15 mins.	− 11·1	+ 4·1	− 8·5	+ 5·9
Next 30 mins.	− 10·5	+ 2·5	− 6·8	+ 5·3
Exp. 23. BW.				
First 30 mins.	− 12·7, − 10·7	+ 2·0, + 2·5	− 14·8, − 11·3	+ 10·0, + 5·5

Exp. 24. P.	Usual medium		Medium containing 4·5 % glucose	
KCl addition ...	None	0·1 *M*	None	0·1 *M*
Q_{O_2} before KCl addition (40 mins.)	− 11·7	− 12·6	− 14·0	− 14·1
Q_{O_2} after KCl addition (40 mins.)	− 13·9	− 28·8	− 10·1	− 14·7

It thus seems to be necessary to have a fairly high concentration of Na ions present before the addition of 0·1 *M* KCl can increase the respiration. In the experiments of Table IX we added 0·1 *M* KCl to brain tissue respiring in media from which the other cations were omitted in turn. From these experiments we draw the following conclusions:

(1) In the absence of all cations but Na, addition of 0·1 *M* KCl provokes no increase in the respiration.

(2) When in addition to Na the usual concentrations of K, or K + Ca or K + Ca + Mg are present, addition of 0·1 *M* KCl increases the respiration. In all three media the respiration is raised to about the same level, but the relative increase is least in Na + K, since the initial respiration in this medium is highest.

(3) When Ca alone is present in addition to the Na, addition of 0·1 *M* KCl causes no increase in the respiration.

(4) It thus seems that the tissue undergoes some change in a medium free from K, as a result of which the respiration can no longer be increased by the addition of 0·1 *M* KCl; and that low concentrations of K, but not of Ca, can prevent this change.

Table IX.

Exp. 25. P.	Na + K		Na + K + Ca + Mg		Na + Ca + K + Mg (1 % glucose)	
KCl addition ...	None	0·1 *M*	None	0·1 *M*	None	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	−19·6	−17·8	−10·9	− 9·8	−10·5	−10·5
Q_{O_2} after KCl addition (30 mins.)	−17·7	−22·8	−12·4	−21·9	−12·1	−21·6

Exp. 26. P.	Na		Na + K		Na + Ca	Na + Ca + K	
KCl addition ...	None	0·1 *M*	None	0·1 *M*	0·1 *M*	None	0·1 *M*
Q_{O_2} before KCl addition (45 mins.)	−14·3	−10·9	−16·0	−18·4	−13·2	−13·1	−13·1
Q_{O_2} after KCl addition (45 mins.)	− 5·5	− 7·4	−13·5	−23·4	− 8·9	−12·7	−21·8

Exp. 27. B.*	Na		Na + K		Na + Ca		Na + Ca + K
KCl addition ...	None	0·1 *M*	None	0·1 *M*	None	0·1 *M*	None
Q_{O_2} before KCl addition (first 20 mins.)	−19·1	−15·8	−19·1	−19·4	−12·2	−10·1	−13·3
Q_{O_2} before KCl addition (second 20 mins.)	−13·8	−11·7	−18·6	−18·8	−11·9	− 8·9	−14·4
Q_{O_2} after KCl addition (40 mins.)	− 7·4	− 9·7	−15·8	−22·4	− 9·2	− 8·4	−14·0

* This experiment was carried out by the original Warburg [1923] technique (low bicarbonate concentration: CO_2 absorption by potash).

Effect of neutral salts on the metabolism of tissues other than brain cortex.

It is important to discover whether the neutral salt effects, which have been observed with brain, can be shown in other surviving tissues; especially since the coloured substances, which produce changes in brain metabolism similar to those produced by neutral salts, also cause these effects in kidney, tumour and other surviving tissues.

In the experiments of Table X, KCl, in 0·1 *M* and lower concentrations, was added to the usual medium, in which slices of various kinds of rat tissue were respiring. The substrate, unless otherwise stated, was glucose. Not once did the addition of KCl provoke an increase of respiration: usually there was a

Table X.

Exp. 28. P. Kidney cortex.

Substrate ...	Glucose		Lactate		
KCl addition ...	None	0·1 *M*	None	0·05 *M*	0·1 *M*
Q_{O_2} before KCl addition (40 mins.)	−20·0	−18·0	−40·9	−39·3	−40·5
Q_{O_2} after KCl addition (40 mins.)	−17·1	− 9·5	−41·1	−33·0	−31·4

Exp. 29. P. Testis.

KCl addition ...	None	0·05 *M*	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	− 9·9	−10·0	−8·4
Q_{O_2} after KCl addition (30 mins.)	−10·0	− 9·2	−7·8

Exp. 30. P. Liver.

KCl addition ...	None	0·05 *M*	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	−10·3	−11·3	−11·0
Q_{O_2} after KCl addition (30 mins.)	−11·3	−11·4	− 9·9

Exp. 31. P. Rat yolk-sac (20 days).

KCl addition ...	None	0·02 *M*	0·05 *M*	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	−12·9	−12·6	−13·0	−13·3
Q_{O_2} after KCl addition (40 mins.)	−11·6	−10·9	− 9·8	− 8·6

Exp. 32. P. Retina.

KCl addition ...	None	0·05 *M*	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	−20·2	−19·0	−19·8
Q_{O_2} after KCl addition (30 mins.)	−19·6	−17·9	−17·4

Exp. 33. P. Jensen rat sarcoma.

KCl addition ...	None	0·05 *M*	0·1 *M*
Q_{O_2} before KCl addition (30 mins.)	−8·2	−8·8	−8·6
Q_{O_2} after KCl addition (30 mins.)	−8·5	−6·6	−5·4

BD. J.R.S.	Usual medium			Usual medium + 0·1 *M* KCl		
	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Exp. 34. 120 mins.	−12·1	+15·8	—	−6·2	+7·6	—
Exp. 35. 100 mins.	−12·3	+18·7	+32·4	−6·8	+8·0	+19·9
	−10·5	+18·2	—	−7·2	+8·5	+19·0

Exp. 36. P. Walker rat carcinoma 256.

	Usual medium	Usual medium + 0·1 *M* KCl
40 mins.	−8·7	−6·1

Exp. 37. BD. J.R.S. In "0·1 *M* KCl medium" part of the sodium chloride was replaced by isotonic KCl to give isotonic medium containing 0·1 *M* KCl.

	Usual medium		"0·1 *M* KCl medium"	
	Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
100 mins.	−9·2	+23·8	−9·2	+15·7
			−7·8	+15·5
Exp. 38. BW. Walker 256. Media as in Exp. 49.				
First 30 mins.	−8·8	+19·6	−7·0	+ 9·8
Second 30 mins.	−8·8	+23·8	−7·2	+10·0

Exp. 39. B. J.R.S. Anaerobic glucolysis.

KCl addition ...	None	0·1 *M*
$Q_G^{N_2}$ before KCl addition (40 mins.)	+31·4	+32·2
$Q_G^{N_2}$ after KCl addition (30 mins.)	+29·4	+22·4

slight fall, the more marked the higher the KCl concentration. We would lay stress on the following:

(1) Testis resembles brain cortex in having a slight aerobic glucolysis.

(2) Rat yolk-sac resembles brain cortex in having a high anaerobic glucolysis and a R.Q. of unity [Dickens and Šimer, 1930, 2].

(3) Retina resembles brain cortex in having a high anaerobic glucolysis and a R.Q. of unity [Dickens and Šimer, 1930, 2], a respiration dependent on the presence of carbohydrate, and a capability of oxidising fructose directly [Dickens and Greville, 1933].

But as none of these tissues suffers an increase of respiration on the addition of 0·1*M* KCl we may conclude that the effect on brain is not associated with any particular metabolic characteristic of that tissue.

(4) Tumour tissues show a marked decrease in aerobic glucolysis, both when 0·1*M* KCl is added to the usual medium and also when part of the NaCl of the medium is replaced by isotonic KCl to give an isotonic medium containing 0·1*M* KCl (Exps. 34–38 inclusive). This effect is in marked contrast to the effect of KCl on brain and to the effect of dinitro-*o*-cresol on both brain and tumour tissue.

The anaerobic glucolysis of tumours is also decreased by the addition of 0·1*M* KCl to the usual bicarbonate medium (Exp. 39).

We have seen that the respiration of brain is very considerably raised when the calcium and magnesium are left out of the usual medium. With the other tissues tested (kidney, testis, tumour) the respiration is unaffected, or insignificantly compared with that of brain, under these conditions (Table XI).

Table XI.

	Na + Ca + K + Mg	Na + K
Exp. 40. P. Kidney.		
Q_{O_2} (first 30 mins.)	−20·8, −21·2	−19·6, −19·0
Q_{O_2} (second 30 mins.)	−21·4, −20·6	−16·8, −15·6
	(Repeated with similar result.)	
Exp. 41. P. Testis.		
Q_{O_2} (first 30 mins.)	−10·1, −10·2	−11·8, −10·4
Q_{O_2} (second 30 mins.)	−10·3, −10·1	−11·6, −10·6
	(Repeated with similar result.)	
Exp. 42. P. J.R.S.		
Q_{O_2} (first 30 mins.)	−7·6, −7·2	−7·0, −7·3
Q_{O_2} (second 30 mins.)	−7·6, −7·3	−7·4, −7·1
	(Repeated with similar result.)	

Table XII.

	Na + Ca + K			Na		
Exp. 43. BW. Testis.	Q_{O_2}	$Q_G^{O_2}$		Q_{O_2}	$Q_G^{O_2}$	
60 mins.	−9·5	+3·7		− 8·4 −10·5	+3·6 +4·3	
Exp. 44. BW. Walker 256.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
First 30 mins.	−18·5	+24·0	+46·0	−17·2	+17·4	+24·4
Second 30 mins.	−21·6	+24·8	+42·4	−16·1	+18·0	+19·6
	(Repeated with similar result.)					
Exp. 45. BW. Mouse yolk-sac.	−12·5	+ 8·7	—	−13·9	+ 8·1	—

We have seen that the aerobic glucolysis of brain is enormously increased when the K, Ca and Mg are left out of the usual medium. The aerobic glucolysis of testis and yolk-sac is unaffected, and that of tumours is slightly decreased, under these conditions (Table XII).

We may therefore say that there is no evidence that any of the effects of neutral salts on the aerobic metabolism of brain tissue, which are described in this paper and that of Ashford and Dixon, are to be observed with slices of any surviving tissue other than brain.

Discussion.

From our results we draw these conclusions. When brain grey matter is respiring in a salt solution, the respiration is steadiest when, in addition to Na, K and Ca are present. K tends to increase the respiration, the bivalent cations Ca and Mg to lower it. Thus, if we leave the Ca out of the Ringer solution, the respiration is higher in the solution containing Na and K only than in the full Ringer solution. The action of the Ca in keeping the respiration small is not overcome by the low concentration of K in the Ringer solution, but it can be overcome by higher concentrations, so that the respiration reaches high values when KCl is added in concentrations of the order of $0 \cdot 1 M$ to the full Ringer solution. When bivalent ions are absent from the medium, the respiration is not a maximum with K present in the concentration in which it is put in Ringer solution; the respiration can be raised further by the addition of $0 \cdot 1 M$ KCl. It may be that the effect of residual bivalent ions in the tissue has to be overcome.

The effect of neutral salts on aerobic glucolysis cannot be easily formulated. Aerobic glucolysis is high when all cations are absent except Na, or when excess K is added to a medium containing Na and Ca. Here the effect of the excess K cannot be regarded as overcoming that of the low concentration of Ca, since the aerobic glucolysis is not high when a low concentration of K is added to the calcium-free medium. The aerobic glucolysis, however, depends on several factors, including, possibly, the respiration. The nature of the Pasteur reaction in muscle, the tissue most studied, is a controversial matter. New ideas on its mechanism in brain have just appeared [Geiger, 1935; see also Dixon, 1935]. It would therefore be unwise to attempt to explain the effect of neutral salts on the aerobic glucolysis at the present time.

Lyotropic effects. We have seen that the acceleration of brain respiration by a high concentration of KCl is associated with no particular substrate and no particular metabolic characteristic of the tissue. We may consider that it is the result of some physical change in the tissue. This is left beyond doubt by our observation that an increase is produced by other cations, the order of activity being:

$$\text{Mg} < \text{Li, Na} < \text{K, Rb, Cs.}$$

This is clearly a lyotropic series. A vast amount of work on many different types of living cell [*v.* Höber, 1926; Gellhorn, 1929] has shown that K has a much greater effect than Li and Na in promoting the swelling of tissues, in increasing the permeability of cell membranes to salts and to cell constituents, in breaking down the normal impermeability of membranes or even in visibly damaging them [*e.g.* Spek, 1921]. Of particular interest is the following. Haldi *et al.* [1927] found that solutions of the alkali chlorides permitted water imbibition by the cerebral hemispheres of the rabbit brain in the order Li, Na < K, Rb, Cs. Thus the swelling of brain tissue and its respiration are increased by alkali cations in the same order. There can be no doubt that the action of these cations on brain respiration results from an alteration by them of the colloidal state of the tissue protoplasm.

Ion antagonism. It is well known that a large number of organisms of most varied type cannot survive in pure solutions of alkali metal salts, but that the addition of a small quantity of the salt of a bivalent metal such as calcium "detoxicates" the solution and permits survival and even growth [for literature *v.* Beutner, 1933; Höber, 1926; Lillie, 1932; Stephenson, 1930]. The apparent "antagonism" of sodium and calcium may be regarded as an expression of their distance apart in the lyotropic series. Roughly we may write:

$$\overset{\xrightarrow{\text{Permeability}}}{\underset{\xrightarrow[\text{Swelling}]{}}{\mathrm{Ca}<\mathrm{Ba}<\mathrm{Mg}<\mathrm{Li}<\mathrm{Na}<\mathrm{Cs}<\mathrm{Rb}<\mathrm{K}}}$$

and say that Ca diffuses slowly, lowers permeability and favours contraction, whilst K diffuses rapidly, increases permeability and favours imbibition. If we start with the tissue in the condition corresponding to one cation, then addition of a cation to the right of the first in the series will cause a swelling and increased permeability, a cation to the left, a contraction and decreased permeability. Thus it is that an "antagonism", somewhat similar to that found between Na and Ca, is shown by Na and K, as in the growth of wheat roots [Osterhout, 1908], hypotonic haemolysis of red cells [Neuschloss, 1920] and the motility of spermatozoa [Gellhorn, 1922].

Neutral salt effects on brain respiration fall in well with these ideas. Ca lowers the respiration, K raises it. In the presence of Na alone, the respiration falls rapidly: the fall is considerably checked by the addition of small quantities of K. As a further expression of the disturbed condition when Na is the only cation, a large aerobic glucolysis appears which is to a great extent removed if K be present. The tissue is in its least deranged state when K and Ca are both present. This state is characterised by a low, steady respiration. Alteration of the colloidal state by removal of Ca or by addition of excess K results in a raised, rather unsteady, respiration, to be regarded as accompanying a deranged condition of the cell.

Na and Ca form a less satisfactory medium than Na and K, since on addition of $0{\cdot}1M$ KCl to the former medium the respiration is not increased. This we cannot explain: the reverse might be expected here, if the controlling factor were simply the salt balance in the solution.

Nature of neutral salt effects. Whilst we have no doubt that the effects of Ca and K on brain respiration are connected with a change in the colloidal state of the protoplasm, the nature of this change remains undecided. It may be a change in the cell membrane, so that the permeability towards reactants or resultants in the respiratory reactions is altered (see for example Warburg's interpretation of his finding that the poisonous action of pure NaCl solution on the ova of *Strongylocentrotus lividus* is exerted through a great increase of the respiration of the eggs [Warburg, 1910]); or it may be a change in the protoplasm affecting the activity of the enzymes, possibly by altering the extent of their active surfaces. As examples of the latter, we may cite the demonstration of ion antagonisms in the effect of neutral salts on the activity of impure invertase solutions [Neuschloss, 1920], and the demonstration by Raab [1927] of a lyotropic series in the inhibitory effect of cations on the oxygen uptake of lysed avian erythrocytes [*cf.* Warburg, 1911].

Anaerobic glucolysis. We have seen that the addition of $0{\cdot}1\,M$ KCl to the usual medium, or the removal of all the cations but Na, leads to a fall in the anaerobic glucolysis, the opposite of the effect on the aerobic glucolysis. This may be explained by an increased permeability in anaerobiosis; it has been shown that anaerobiosis renders muscle membranes more permeable to chloride [Winterstein and Hirschberg, 1927; see also Gerard, 1932, for nerve]. Then $0{\cdot}1\,M$ KCl, which in aerobiosis is favourable to metabolism, becomes on account of increased diffusibility toxic in anaerobiosis. Again, when the K and Ca are left out of the Ringer solution, the increased permeability which results is favourable to aerobic metabolism, but, since the permeability is already raised by anaerobiosis, unfavourable to anaerobic metabolism. Anaerobic glucolysis by tumour as well as brain decreases when Ca and K are removed from the medium [Lasnitzki, 1933; Lasnitzki and Rosenthal, 1933; see also Exp. 44].

Anions. In most neutral salt actions, the action of the cations is much modified by the nature of the anions [*e.g.* Pauli, 1903; Mathews, 1904; Overton, 1904; Kahho, 1921]. Thiocyanate and iodide are in general similar in their effects to K and promote its action. We have however found that $0{\cdot}05$–$0{\cdot}1\,M$ KI and KCNS in their effect on brain respiration differ little from KCl of the same concentration, and that NaCNS has no greater action than NaCl. In this indifference to anions, brain resembles nerve but is in contrast to intact muscle [Chang *et al.*, 1935].

Other tissues. We have seen that the effect of addition of excess KCl on the respiration of brain is in great contrast to the effect on the respiration of all the other surviving tissues tested. The same is true of the effect of Ca removal on the respiration, and of Ca and K removal on the aerobic glucolysis. Our observations on kidney agree with those of Siebeck [1912], who found that the respiration of whole frog kidneys *in vitro* is slightly decreased by the addition of KCl.

Of all the tissues, intact muscle seems to be the only one which resembles brain in the way in which the respiration is affected by KCl. Claude Bernard observed that muscles become non-excitable in KCl. Frog muscle can become non-excitable when immersed in Ringer solution containing only three times the normal amount of K: lowering the Ca has the same effect [Sereni, 1925]. K also produces a contracture [Ringer, 1886]. The contracture and loss of irritability are accompanied by increased oxygen uptake, whilst with the higher concentrations of K glycolysis appears [Fenn, 1931; Hegnauer *et al.*, 1934]. It seems, however, that K effects on intact muscle are hardly to be compared with those on brain slices. According to Fenn [1931], there is "strong evidence in favour of the view that chemical stimuli act through the normal contractile mechanism"; and Chang *et al.* [1935] go so far as to say that the increase of muscle respiration caused by KCl "is most probably due to the contracture induced by this salt, and the fundamental effect may be a depression. This is at least the case for heart muscle, the respiration (and tone) of which is decreased by excess potassium, as in nerve".

One is tempted to suggest that the peculiar behaviour of brain tissue is at any rate in part due to its lipin nature: this is its chief difference from retina, for example. Höber [1926] suggested that a state of dispersion between lyophile and lyophobe is the most favourable for ion antagonisms; whilst Schürmeyer [1925] found that the activity of purified invertase was not ordinarily subject to salt effects, but became so when lecithin, but not gelatin or albumin, was added.

Whether this hypothesis be acceptable or no, we cannot escape the conclusion that the metabolism of brain tissue is subject to control by alterations in the medium in a way that is not open to most other surviving tissues, and that this control bears a remarkable resemblance to the control of muscle contraction.

SUMMARY.

1. Following the observation of Ashford and Dixon that the respiration and aerobic glucolysis of rabbit brain slices are increased by the addition of $0{\cdot}1\,M$ KCl to the medium, a study has been made of the effect of neutral salts on the metabolism in salt solutions of brain and other surviving tissues.

2. The results of Ashford and Dixon have been confirmed with rat brain, and rat tissues have been used throughout.

3. $0{\cdot}1\,M$ KCl added to the medium increases the respiration of brain tissue whether glucose, lactate, pyruvate or fructose is the substrate. The increased glucose oxidation is complete.

4. All the alkali metal chlorides increase the respiration of brain tissue when added in $0{\cdot}1\,M$ concentration to the medium. The order of activity is

$$\text{Li, Na} < \text{Rb, Cs, K.}$$

5. The effect on brain metabolism of leaving the various cations in turn out of the medium has been tested:

(*a*) Ca and Mg lower the respiration, K raises it.

(*b*) In the presence of Ca, K and Na the respiration is low and steady.

(*c*) In the presence of K and Na the respiration is high and not very steady.

(*d*) When Na is the only cation the respiration is initially high but falls rapidly.

(*e*) When Na is the only cation there is a high aerobic glucolysis, which does not appear when Ca and K are added.

6. In agreement with Ashford and Dixon, it is found that $0{\cdot}1\,M$ KCl added to the medium decreases the anaerobic glucolysis. When Na is the only cation, increased aerobic glucolysis is again accompanied by decreased anaerobic glucolysis. It is suggested that these effects are due to increased cell permeability in anaerobiosis.

7. The conditions in the media which produce large changes in brain metabolism do not produce such effects on any other surviving normal or tumour tissue tested. In these:

(*a*) $0{\cdot}1\,M$ KCl added to the medium never causes a rise of respiration or aerobic glucolysis. Usually a fall is observed.

(*b*) The respiration is no higher in presence of K and Na than in presence of Na, Ca and K.

(*c*) When Na is the only cation there is no increased aerobic glucolysis.

8. The effects of neutral salts on brain metabolism are ascribed to changes in the colloidal state of the protoplasm. The similarity between the control of brain metabolism by changes in the medium and the control of muscle contraction is pointed out.

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REFERENCES.

Ashford and Dixon (1935). *Biochem. J.* **29**, 157.

Beutner (1933). Physical chemistry of living tissues and life processes. (Baillière, Tindall and Cox, London.)

Chang, Shaffer and Gerard (1935). *Amer. J. Physiol.* **111**, 681.

Dickens (1934). *Nature*, **134**, 382.

—— (1935). *Nature*, **135**, 762.

—— and Greville (1933). *Biochem. J.* **27**, 832.

—— and Šimer (1930, 1). *Biochem. J.* **24**, 905.

—— —— (1930, 2). *Biochem. J.* **24**, 1301.

—— —— (1931). *Biochem. J.* **25**, 975.

Dixon (1935). *Biochem. J.* **29**, 973.

Dodds and Greville (1934). *Lancet*, **1**, 398.

Fenn (1931). *J. Pharm. Exp. Ther.* **42**, 81.

Friedemann and Graeser (1933). *J. Biol. Chem.* **100**, 291.

Geiger (1935). *Biochem. J.* **29**, 811.

Gellhorn (1922). *Pflüger's Arch.* **193**, 576.

—— (1929). Das Permeabilitätsproblem. (Springer, Berlin.)

Gerard (1932). *Physiol. Rev.* **12**, 537.

Haldi, Rauth, Larkin and Wright (1927). *Amer. J. Physiol.* **80**, 631.

Hegnauer, Fenn and Cobb (1934). *J. Cell. Comp. Physiol.* **4**, 505.

Höber (1926). Physikalische Chemie der Zelle und der Gewebe. 6th ed. (Engelmann, Leipzig.)

Kahho (1921). *Biochem. Z.* **123**, 284.

Krebs and Henseleit (1932). *Z. physiol. Chem.* **210**, 33.

Lasnitzki (1933). *Biochem. Z.* **264**, 285.

—— and Rosenthal (1933). *Biochem. Z.* **262**, 203.

Lillie (1932). Protoplasmic action and nervous action. 2nd ed. (University of Chicago Press.)

Loeb (1913). *Science*, **37**, 427.

Mathews (1904). *Amer. J. Physiol.* **11**, 455.

Neuschloss (1920). *Pflüger's Arch.* **181**, 17.

Osterhout (1908). *Jahrb. wissensch. Botan.* **46**, 121.

Overton (1904). *Pflüger's Arch.* **105**, 176.

Pauli (1903). *Hofmeister's Beitr.* **3**, 225.

Raab (1927). *Pflüger's Arch.* **217**, 124.

Ringer (1886). *J. Physiol.* **7**, 291.

Schürmeyer (1925). *Pflüger's Arch.* **208**, 595.

Sereni (1925). *J. Physiol.* **60**, 1.

Siebeck (1912). *Pflüger's Arch.* **148**, 443.

Spek (1921). *Acta Zoologika*, **2**, 153.

Stephenson (1930). Bacterial metabolism. (Longmans, Green and Co., London.)

Warburg (1910). *Z. physiol. Chem.* **66**, 305.

—— (1911). *Z. physiol. Chem.* **70**, 413.

—— (1923). *Biochem. Z.* **142**, 317.

—— (1924). *Biochem. Z.* **152**, 51.

—— (1926). Über den Stoffwechsel der Tumoren. (Springer, Berlin.)

—— (1929). *Biochem. Z.* **204**, 482.

Winterstein and Hirschberg (1927). *Pflüger's Arch.* **217**, 216.

CLXXVIII. THE OPTICAL PROPERTIES OF SOME BILE ACIDS.

BY BERTIL JOSEPHSON.

From the Chemical Department of the Caroline Institute, Stockholm.

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THE rotatory power of the bile acids has acquired a new practical interest in connection with the method of Jenke [1928] for their determination in bile. For most of these acids and their sodium salts the optical rotations have been determined by several investigators, but except for one [Hoppe-Seyler, 1863] all have used only sodium light. In many cases the figures given in the different publications also show quite large differences.

The principle of Jenke's method is, (1) hydrolysis of the conjugated bile acids with strong alkalis, (2) extraction with ether-light petroleum of the free acids precipitated by acidification, and (3) polarimetric determination of the extracted unconjugated acids in alcoholic solution. This alcoholic solution however usually contains a considerable amount of bile pigments, which are optically inactive but make polarimetric reading in sodium light difficult or impossible. As will be shown in a later publication it is even possible in many cases by selection of light of suitable wave length to make the polarimetric determination of the bile acids as salts in the alkaline hydrolysis solution without the time-consuming acid extraction.

The determination of the rotatory dispersion of the common bile acids is also of interest as an addition to other well-known and characteristic physical data for these substances; in particular it seemed desirable to compare the specific rotations and rotatory dispersions of the bile acids and their salts in different solvents.

An investigation of the rotatory dispersions of glycocholic, taurocholic and cholic acids and their salts was made by Hoppe-Seyler [1863]: his substances however were probably impure, as will be seen from the description of their preparation, and his polarimetric apparatus was rather primitive. His values were thus very irregular and do not agree well with those found by later investigators including myself.

My determinations were made in a Schmidt and Haentsch Lippich-polarimeter readable to 0·01° and with a Hoffmann monochromator. Half-shadow angle 5°. The instrument was tested at the various wave lengths with a controlled quartz prism. The temperature was 20 ± 1°. All observations were made in 4 dm. tubes.

The following wave lengths were used: 6863 (B), 6563 (C), 5893 (D), 5463 (Hg), 4861 (F) and 4388 (Hg) Å.

The substances examined were the same as in earlier work, where description of the preparations and analyses can be found [Josephson, 1933]. Cholic, deoxycholic, glycocholic and taurocholic acids and their sodium and potassium salts were investigated.

The salts were examined in aqueous solution of 0·5, 1, 2·5, 5 (and 15) % concentration calculated on the bile acids. The cholates and deoxycholates were also tested in KCl solutions and in excess of sodium and potassium hydroxides of various concentrations. The free acids were examined in alcoholic solutions of various concentrations, depending on their solubilities. Cholic and deoxycholic acids were also examined in solution in acetic acid, acetone, chloroform, benzene and ether. The solubilities in benzene and ether are however so low that no satisfactory values could be obtained, and these observations are therefore not recorded.

The specific rotations and dispersion coefficients found, $\frac{[\alpha]_\lambda}{[\alpha]_{6867}}$, are shown in the tables.

For *cholic acid* in alcoholic solution Hoppe-Seyler found $[\alpha]_D$ 33·9°, Pregl [1910] 35·1° and Vahlen [1895] 37·0° independent of the concentration. Pregl's value agrees very well with mine ($[\alpha]_D$ 35·07°, Table I). In acetic acid and acetone the rotation and dispersion were very similar to those in alcohol, but in chloroform the rotation was much less, and the dispersion larger.

Table I.

Cholic acid.

Solvent ...	Alcohol		Acetic acid		Acetone		Chloroform	
c	0·432–1·440		0·5–5·0		0·277–1·107		0·197 %	
	$[\alpha]$	d	$[\alpha]$	d	$[\alpha]$	d	$[\alpha]$	d
6867 λ	25·4	—	26·70	—	28·13	—	15·77	—
6563	27·78	1·09	29·21	1·09	30·71	1·09	17·77	1·13
5893	35·07	1·38	37·52	1·40	37·94	1·35	24·11	1·53
5463	41·67	1·64	45·60	1·69	45·88	1·63	30·46	1·93
4861	55·03	2·17	59·19	2·22	60·52	2·15	45·69	2·90
4388	73·44	2·89	77·51	2·89	79·04	2·81	71·07	4·51

In this and following tables c = g./100 ml. solution and $d = \frac{[\alpha]_\lambda}{[\alpha]_{6867}}$.

For Na cholate in water from 30 to 9 % concentration Hoppe-Seyler found $[\alpha]_D$ 26·0–28·5° and for K cholate $[\alpha]_D$ 27·2–31·8°. Vahlen found the rotation of a 4 % solution of the Na salt to be $[\alpha]_D$ 27·6° and of a 3 % solution of K salt $[\alpha]_D$ 27·0–27·6°. My values (Table II) coincide fairly well with those of Vahlen but not with those of Hoppe-Seyler. I found the optical activity of the Na and K salts dissolved in water to be very similar, the latter being about 1–2 % lower than the former. For want of space the K values therefore are not recorded. Between 15 and 5 % the activity of the salts seemed to be independent of the concentration, but below 5 % the activity increased with decreasing concentration, and below about 1·5 % this increase became rather large. It therefore seems as if there were some alteration in the molecular state at this concentration limit. The rotatory dispersion of the salts was independent of the concentration and very similar to that of the free acid. As shown in Table III the activity was only very slightly changed by the presence of other electrolytes (KOH, KCl, NaOH).

Table II.

Na cholate in water. $[\alpha]$

c	15	5	2·5	1	0·5	d
6867 λ	21·05	21·10	21·50	22·50	23·50	—
6563	22·75	22·80	23·30	24·25	25·00	1·08
5893	28·80	28·85	29·50	31·00	32·50	1·37
5463	34·18	34·25	35·10	37·50	39·50	1·65
4861	45·68	45·80	46·80	49·75	50·50	2·18
4388	57·62	57·75	59·50	62·75	66·00	2·78

Table III.

K cholate, *c* = 2·5. [α]

Solvent ...	Water	KOH 0·5 *N*	KOH 1·0 *N*	KCl 1·0 *M*	KCl 1·7 *M*
6867 λ	21·50	21·40	21·40	21·40	21·40
6563	23·40	23·30	23·30	23·40	23·30
5893	29·70	29·70	29·40	29·50	29·40
5463	35·20	35·70	34·70	34·90	34·70
4861	46·90	46·40	46·70	46·90	46·70
4388	59·80	59·60	59·20	59·40	59·20

Table IV.

Deoxycholic acid.

Solvent ...	Alcohol		Acetic acid		Acetone		Chloroform	
c	0·5–5·0		0·212–0·849		0·240–0·960		0·213–0·485	
	[α]	*d*	[α]	*d*	[α]	*d*	[α]	*d*
6867 λ	40·91	—	40·47	—	40·63	—	32·11	—
6563	44·30	1·08	44·17	1·09	43·49	1·07	35·29	1·10
5893	55·56	1·36	55·06	1·36	55·99	1·38	44·71	1·39
5463	65·49	1·60	66·25	1·64	66·15	1·63	52·94	1·65
4861	86·60	2·12	87·16	2·15	86·72	2·13	74·12	2·31
4388	109·85	2·69	110·49	2·73	110·68	2·72	117·64	3·66

For *deoxycholic acid* (Table IV) the following figures for $[\alpha]_D$ have been recorded: Langheld [1908] 53·28°; Vahlen [1895] 49·86°; Pregl [1910] 51·86°; Fischer [1911] 55·20°; Wieland and Sorge [1916] 57·02°. No examination of its rotatory dispersion seems to have been published. I found the specific rotation as well as the rotatory dispersion to be almost identical in alcoholic, acetic acid and acetone solutions. As for cholic acid the specific rotation was less and the dispersion higher in chloroform than in alcoholic solution. For this acid also the rotation and the dispersion were rather similar to those of the Na (Table V) and the K (Table VI) salts in water, the K salt being about 2–4 % more active than the Na salt. As for the cholates the specific rotation increased with decreasing

Table V.

Na deoxycholate in water. [α]

c	5	2·5	1	0·5	*d*
6867 λ	32·70	32·90	34·25	38·00	—
6563	35·90	36·10	37·50	41·50	1·10
5893	45·40	45·70	47·50	52·50	1·39
5463	53·70	54·00	56·00	62·00	1·64
4861	69·25	69·60	72·50	80·50	2·12
4388	88·30	89·00	93·75	104·00	2·72

Table VI.

Deoxycholates, *c* = 1. [α]

Salt ...	Na	Na	Na	K	K	K	K
Solvent ...	Water	NaOH 0·2 *N*	NaOH 0·4 *N*	Water	KOH 0·5 *N*	KCl 0·25 *M*	KCl 0·75 *M*
6867 λ	34·25	32·50	30·75	35·50	30·00	32·50	28·00
6563	37·50	35·75	34·00	39·00	32·75	35·50	30·75
5893	47·50	45·00	42·50	49·25	41·50	44·50	40·25
5463	56·00	53·25	50·50	58·00	46·75	53·00	48·50
4861	72·50	68·75	65·25	74·00	63·75	68·50	60·00
4388	93·75	89·00	84·50	96·25	—	—	—

concentration, and this increase was much more rapid below a concentration of about 2 %, indicating some change in the molecule. The dispersions were very similar for the free acid and the alkali salts, and somewhat less than that of cholic acid. The deoxycholates are much more sensitive to other electrolytes in the solution than the cholates; it seems however as if the decrease in optical activity caused by electrolytes depends more on their concentration than on their chemical nature (Table VI), as it is approximately proportional to the molecular concentration of the added electrolyte.

Glycocholic acid and its sodium salt have been investigated by Hoppe-Seyler and by Letsche [1909]. The former found $[\alpha]_D$ for the free acid in alcoholic solution to be 29·0° and for the Na salt in water 21·8°, both independent of the concentration. Letsche found for the free acid $[\alpha]_D$ 32·3° and for the salt in 8·5 % aqueous solution 24·5°. The figures of Letsche agree well with mine. I did not, as for cholic and deoxycholic acids, find the rotation of the free acid in alcohol to be independent of the concentration; there was a slight increase in activity with decreasing concentration. Even the rotatory dispersion increased slightly with decreasing concentration (Table VII). The Na and K salts in water also showed an increase at decreasing concentration, which was obviously larger below about 1·75 %. The activities of the Na and K salts were almost identical, and the dispersions seemed to be nearly independent of the concentration (Table VIII). The dispersion was also somewhat larger for the free acid than for the salts.

Table VII.

Glycocholic acid in alcohol.

c	10·0		5·0		2·5		1·0		0·5	
	[α]	*d*	[α]	*d*	[α]	*d*	[α]	*d*	[α]	*d*
6867 λ	22·55	—	22·60	—	22·60	—	22·75	—	22·75	—
6563	25·40	1·13	25·50	1·13	25·50	1·13	25·50	1·13	25·50	1·12
5893	31·85	1·41	32·30	1·43	32·50	1·44	33·50	1·48	33·50	1·48
5463	37·75	1·67	38·25	1·69	38·80	1·72	39·50	1·74	40·00	1·77
4861	49·05	2·18	49·75	2·20	50·40	2·23	51·50	2·27	52·00	2·30
4388	63·10	2·80	64·30	2·85	65·80	2·91	67·75	2·99	69·00	3·03

Table VIII.

Na glycocholate in water. [α]

c	15·0	5·0	2·5	1·0	0·5	*d*
6867 λ	16·93	18·90	20·10	23·00	25·00	—
6563	18·88	20·90	22·20	25·00	27·00	1·09
5893	24·29	26·35	27·80	32·00	34·00	1·38
5463	28·31	31·35	32·90	37·50	40·50	1·64
4861	36·82	40·80	43·40	49·50	52·50	2·14
4388	48·82	53·85	56·70	66·00	73·00	2·86

Taurocholic acid has been examined by Kazumo and Yamazaki [1934], who found $[\alpha]_D$ 38·8° in diluted alcohol, but this value was calculated from one reading ($\alpha = +0{\cdot}2°$), and they do not mention the concentration of the alcohol. The specific rotation of the Na salt was measured by Hoppe-Seyler, who found $[\alpha]_D$ in water 22·4° independent of the concentration, and by Hammarsten [1909], who found $[\alpha]_D$ 23·27–24·14° between 2·32 and 5·42 %. Tanaka [1933] found $[\alpha]_D$ 23·87° ($c = 1{\cdot}28$), Makimo [1934] 24·44° ($c = 0{\cdot}9$) and Kazumo and Yamazaki 23·9° in diluted alcohol (from one value $\alpha = 0{\cdot}1°$). For the free acid, dissolved in alcohol, I found, as for glycocholic acid, slightly increasing specific rotation and rotatory dispersion with decreasing concentration (Table IX). The Na salt in

Table IX.

Taurocholic acid in alcohol.

c	1·64		0·82		0·41	
	[α]	d	[α]	d	[α]	d
6867 λ	21·43	—	21·62	—	21·77	—
6563	23·08	1·08	23·05	1·07	23·17	1·06
5893	28·74	1·34	29·21	1·35	29·49	1·36
5463	33·20	1·55	33·97.	1·57	34·46	1·58
4861	43·80	2·04	45·19	2·09	46·34	2·13
4388	65·15	3·04	66·68	3·08	68·29	3·14

Table X.

Na taurocholate in water. [α]

c	5·0	2·5	1·25	0·625	0·33	d
6867 λ	16·05	16·40	16·80	18·20	20·33	—
6563	17·40	17·70	18·00	19·60	21·96	1·08
5893	21·65	22·10	22·60	24·80	28·03	1·36
5463	25·70	26·60	27·30	29·60	33·12	1·62
4861	35·05	35·60	36·40	39·30	43·85	2·17
4388	70·65	72·50	75·60	84·10	90·73	4·44

water also showed increasing activity with decreasing concentration, and this increase was larger below about 0·75 % (Table X). The rotatory dispersion of the sodium salt seemed to be independent of the concentration; it was much larger than that of the free acid.

None of the substances examined satisfied the Lommel or the Drude formula, so their rotatory dispersions may be characterised as complex. As the deviation in most cases was rather large, the calculated values are not given.

Summary.

The specific rotation and rotatory dispersion were determined for the following bile acids and their alkali salts: cholic, deoxycholic, glycocholic and taurocholic acids.

For cholic and deoxycholic acids the specific rotations were very similar in alcohol, acetone and acetic acid, and the rotatory dispersions were nearly identical in all these solvents and for the alkali salts in water. In chloroform the rotation was lower and the dispersion higher. The rotatory dispersions of these two acids were very similar.

The specific rotations of the sodium and potassium salts showed only minimum differences. The optical activity of the salts of cholic acid was scarcely influenced by other electrolytes in the solution, but that of the deoxycholic salts was obviously decreased.

In alcoholic solution glycocholic and taurocholic acids have higher specific rotations at low concentrations than at higher. Their dispersions were somewhat similar to those of the unconjugated acids.

It was found that in aqueous solution the alkali salts of all the acids examined showed increasing optical activity at decreasing concentration. This increase was much larger below a certain critical concentration, which was different for the different acids; for taurocholic acid it was about 0·75 % and for the others between 1·5 and 2·0 %. This change in the rate of increase may possibly be due to some alteration in the state of the bile acid ion.

REFERENCES.

Fischer (1911). *Z. physiol. Chem.* **73**, 233.
Hammarsten (1909). *Z. physiol. Chem.* **61**, 454.
Hoppe-Seyler (1863). *J. prakt. Chem.* **89**, 257.
Jenke (1928). *Arch. exp. Path. Pharm.* **130**, 280.
Josephson (1933). *Biochem. Z.* **263**, 428.
Kazumo and Yamazaki (1934). *Z. physiol. Chem.* **224**, 160.
Langheld (1908). *Ber. deutsch. chem. Ges.* **41**, 381.
Letsche (1909). *Z. physiol. Chem.* **60**, 462.
Makimo (1934). *J. Biochem.* **19**, 249.
Pregl (1910). *Z. physiol. Chem.* **65**, 158.
Tanaka (1933). *Z. physiol. Chem.* **220**, 39.
Vahlen (1895). *Z. physiol. Chem.* **21**, 273.
Wieland and Sorge (1916). *Z. physiol. Chem.* **97**, 1.

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